

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
30 May 2002 (30.05.2002)

PCT

(10) International Publication Number
WO 02/42418 A2(51) International Patent Classification⁷: C12N

(21) International Application Number: PCT/US01/43607

(22) International Filing Date:
20 November 2001 (20.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/252,123 20 November 2000 (20.11.2000) US
60/285,478 20 April 2001 (20.04.2001) US
60/306,727 20 July 2001 (20.07.2001) US
60/317,845 7 September 2001 (07.09.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US 60/252,123 (CON)
Filed on 20 November 2000 (20.11.2000)
US 60/285,478 (CON)
Filed on 20 April 2001 (20.04.2001)
US 60/306,727 (CON)
Filed on 20 July 2001 (20.07.2001)
US 60/317,845 (CON)
Filed on 7 September 2001 (07.09.2001)

(US). SELIFONOVA, Olga, V. [RU/US]; 1405 Olive Lane N. #318, Plymouth, MN 55447 (US). JESSEN, Holly [US/US]; 6618 Brenden Court, Chanhassen, MN 55317 (US). GORT, Steven, J. [US/US]; 3207 Quarles Road, Brooklyn Park, MN 55429 (US). SELMER, Thorsten [DE/DE]; Cappeler Strasse 12, 35039 Marburg (DE). BUCKEL, Wolfgang [DE/DE]; Am Koeppel 8, 35043 Marburg (DE).

(74) Agent: DEGRANDIS, Paula; Cargill, Incorporated, P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (for all designated States except US):
CARGILL, INCORPORATED [US/US]; 15407
McGinty Road West, Wayzata, MN 55391-2399 (US).

Published:

— without international search report and to be republished upon receipt of that report

(72) Inventors; and

(75) Inventors/Applicants (for US only): GOKARN, Ravi, R.
[—/US]; 3205 Harbor, Lane #4311, Plymouth, MN 55447

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/42418 A2

(54) Title: 3-HYDROXYPROPIONIC ACID AND OTHER ORGANIC COMPOUNDS

(57) Abstract: The invention provides methods and materials related to producing 3-HP as well as other organic compounds. Specifically, the invention provides isolated nucleic acids, polypeptides, host cells, and methods and materials for producing 3-HP and other organic compounds.

BEST AVAILABLE COPY

5

3-HYDROXYPROPIONIC ACID AND OTHER ORGANIC COMPOUNDS

FIELD OF THE INVENTION

The invention relates to enzymes and methods that can be used to produce organic
10 acids and related products.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from the following U.S. Provisional Patent
Applications, which are herein incorporated by reference: U.S. Provisional Patent
15 Application Serial Number 60/252,123, filed November 20, 2000; U.S. Provisional Patent
Application Serial Number 60/285,478, filed April 20, 2001; U.S. Provisional Patent
Application Serial Number 60/306,727, filed July 20, 2001; and U.S. Provisional Patent
Application Serial Number 60/317,845, filed September 7, 2001.

20

BACKGROUND

Organic chemicals such as organic acids, esters, and polyols can be used to
synthesize plastic materials and other products. To meet the increasing demand for
organic chemicals, more efficient and cost effective production methods are being
developed which utilize raw materials based on carbohydrates rather than hydrocarbons.
25 For example, certain bacteria have been used to produce large quantities of lactic acid
used in the production of polylactic acid.

3-hydroxypropionic acid (3-HP) is an organic acid. Although several chemical
synthesis routes have been described to produce 3-HP, only one biocatalytic route has
been heretofore previously disclosed (WO 01/16346 to Suthers, et al.). 3-HP has utility
30 for specialty synthesis and can be converted to commercially important intermediates by
known art in the chemical industry, e.g., acrylic acid by dehydration, malonic acid by

oxidation, esters by esterification reactions with alcohols, and reduction to 1,3 propanediol.

SUMMARY

5 The invention relates to methods and materials involved in producing 3-hydroxypropionic acid and other organic compounds (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, esters of 3-HP, and malonic acid and its esters). Specifically, the invention provides nucleic acid molecules, polypeptides, host cells, and methods that can be used to produce 3-HP and other organic
10 compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, esters of 3-HP, and malonic acid and its esters. 3-HP has potential to be both biologically and commercially important. For example, the nutritional industry can use 3-HP as a food, feed additive or preservative, while the derivatives mentioned above can be produced from 3-HP. The nucleic acid molecules described herein can be
15 used to engineer host cells with the ability to produce 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP. The polypeptides described herein can be used in cell-free systems to make 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and
20 esters of 3-HP. The host cells described herein can be used in culture systems to produce large quantities of 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP.

One aspect of the invention provides cells that have lactyl-CoA dehydratase
25 activity and 3-hydroxypropionyl-CoA dehydratase activity, and methods of making products such as those described herein by culturing at least one of the cells that have lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity. In some embodiments, the cell can also contain an exogenous nucleic acid molecule that encodes one or more of the following polypeptides: a polypeptide having E1 activator
30 activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-

CoA dehydratase activity; and a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. Additionally, the cell can have CoA transferase activity, CoA synthetase activity, poly hydroxyacid synthase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, and/or lipase activity.

- 5 Moreover, the cell can contain at least one exogenous nucleic acid molecule that expresses one or more polypeptides that have CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, CoA synthetase activity, poly hydroxyacid synthase activity, and/or lipase activity.

- In another embodiment of the invention, the cell that has lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity produces a product, for example, 3-HP, polymerized 3-HP, and/or an ester of 3-HP, such as methyl hydroxypropionate, ethyl hydroxypropionate, propyl hydroxypropionate, and/or butyl hydroxypropionate. Accordingly, the invention also provides methods of producing one or more of these products. These methods involve culturing the cell that has lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity under conditions that allow the product to be produced. These cells also can have CoA synthetase activity and/or poly hydroxyacid synthase activity.

- Another aspect of the invention provides cells that have CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity. In some embodiments, these cells also can contain an exogenous nucleic acid molecule that encodes one or more of the following polypeptides: a polypeptide having E1 activator activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; a polypeptide having CoA synthetase activity; and a polypeptide having poly hydroxyacid synthase activity.

In another embodiment of the invention, the cell that has CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity can produce a product, for example, polymerized acrylate.

- Another aspect of the invention provides a cell comprising CoA transferase activity, lactyl-CoA dehydratase activity, and lipase activity. In some embodiments, the cell also can contain an exogenous nucleic acid molecule that encodes one or more of the

following polypeptides: a polypeptide having CoA transferase activity; a polypeptide having E1 activator activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; and a polypeptide having lipase activity. This
5 cell can be used, among other things, to produce products such as esters of acrylate (e.g., methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate).

In some embodiments, 1,3 propanediol can be created from either 3-HP-CoA or 3-HP via the use of polypeptides having enzymatic activity. These polypeptides can be used either *in vitro* or *in vivo*. When converting 3-HP-CoA to 1,3 propanediol,
10 polypeptides having oxidoreductase activity or reductase activity (e.g., enzymes from the 1.1.1.- class of enzymes) can be used. Alternatively, when creating 1,3 propanediol from 3-HP, a combination of (1) a polypeptide having aldehyde dehydrogenase activity (e.g., an enzyme from the 1.1.1.34 class) and (2) a polypeptide having alcohol dehydrogenase activity (e.g., an enzyme from the 1.1.1.32 class) can be used.

15 In some embodiments of the invention, products are produced *in vitro* (outside of a cell). In other embodiments of the invention, products are produced using a combination of *in vitro* and *in vivo* (within a cell) methods. In yet other embodiments of the invention, products are produced *in vivo*. For methods involving *in vivo* steps, the cells can be isolated cultured cells or whole organisms such as transgenic plants, non-
20 human mammals, or single-celled organisms such as yeast and bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells). Hereinafter such cells are referred to as production cells. Products produced by these production cells can be organic products such as 3-HP and/or the nucleic acid molecules and polypeptides described herein.

25 Another aspect of the invention provides polypeptides having an amino acid sequence that (1) is set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161, (2) is at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161, (3) has at least 65 percent sequence identity with at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2,
30 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161, (4) is a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 having conservative amino acid substitutions,

or (5) has at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. Accordingly, the invention also provides nucleic acid sequences that encode any of the polypeptides described herein as well as specific binding agents that bind to any of the polypeptides described herein. Likewise, the invention provides transformed cells that contain any of the nucleic acid sequences that encode any of the polypeptides described herein. These cells can be used to produce nucleic acid molecules, polypeptides, and organic compounds. The polypeptides can be used to catalyze the formation of organic compounds or can be used as antigens to create specific binding agents.

10 In yet another embodiment, the invention provides isolated nucleic acid molecules that contain at least one of the following nucleic acid sequences: (1) a nucleic acid sequence as set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; (2) a nucleic acid sequence having at least 10 consecutive nucleotides from a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, 15 or 163; (3) a nucleic acid sequences that hybridize under hybridization conditions (e.g., moderately or highly stringent hybridization conditions) to a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; (4) a nucleic acid sequence having 65 percent sequence identity with at least 10 consecutive nucleotides from a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; and (5) a nucleic acid sequence having at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. Accordingly, the invention also provides a production cell that contains at least one exogenous nucleic acid having any the nucleic acid sequences provided above. The production cell can be used to express polypeptides that have an enzymatic activity such as CoA transferase activity, lactyl-CoA dehydratase activity, CoA synthase activity, dehydratase activity, dehydrogenase activity, malonyl CoA reductase activity, β -alanine ammonia lyase activity, and/or 3-hydroxypropionyl-CoA dehydratase activity. Accordingly, the invention also provides methods of producing polypeptides encoded by the nucleic acid sequences described above.

30 The invention also provides several methods such as methods for making 3-HP from lactate, phosphoenolpyruvate (PEP), or pyruvate. In some embodiments, methods

for making 3-HP from lactate, PEP, or pyruvate involve culturing a cell containing at least one exogenous nucleic acid under conditions that allow the cell to produce 3-HP. These methods can be practiced using the various types of production cells described herein. In some embodiments, the production cells can have one or more of the following activities: CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, dehydratase activity, and/or malonyl CoA reductase activity.

In other embodiments, the methods involve making 3-HP wherein lactate is contacted with a first polypeptide having CoA transferase activity or CoA synthetase activity such that lactyl-CoA is formed, then contacting lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, then contacting acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA, and then contacting 3-hydroxypropionic acid-CoA with the first polypeptide to form 3-HP or with a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form 3-HP.

Another aspect of the invention provides methods for making polymerized 3-HP. These methods involve making 3-hydroxypropionic acid-CoA as described above, and then contacting the 3-hydroxypropionic acid-CoA with a polypeptide having polyhydroxyacid synthase activity to form polymerized 3-HP.

In yet another embodiment of the invention, methods for making an ester of 3-HP are provided. These methods involve making 3-HP as described above, and then additionally contacting 3-HP with a fifth polypeptide having lipase activity to form an ester.

The invention also provides methods for making polymerized acrylate. These methods involve culturing a cell that has both CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity such that polymerized acrylate is made. Accordingly, the invention also provides methods of making polymerized acrylate wherein lactate is contacted with a first polypeptide having CoA synthetase activity to form lactyl-CoA, then contacting lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, and then

contacting acrylyl-CoA with a third polypeptide having poly hydroxyacid synthase activity to form polymerized acrylate.

The invention also provides methods of making an ester of acrylate. These methods involve culturing a cell that has CoA transferase activity, lipase activity, and
5 lactyl-CoA dehydratase activity under conditions that allow the cell to produce an ester.

In another embodiment, the invention provides methods for making an ester of acrylate, wherein acrylyl-CoA is formed as described above, and then acrylyl-CoA is contacted with a polypeptide having CoA transferase activity to form acrylate, and acrylate is contacted with a polypeptide having lipase activity to form the ester.

10 The invention also provides methods for making 3-HP. These methods involve culturing a cell containing at least one exogenous nucleic acid that encodes at least one polypeptide such that 3-HP is produced from acetyl-CoA or malonyl-CoA.

Alternative embodiments provide methods of making 3-HP, wherein acetyl-CoA is contacted with a first polypeptide having acetyl-CoA carboxylase activity to form
15 malonyl-CoA, and malonyl-CoA is contacted with a second polypeptide having malonyl-CoA reductase activity to form 3-HP.

In other embodiments, malonyl-CoA can be contacted with a polypeptide having malonyl-CoA reductase activity so that 3-HP can be made.

In another embodiment, the invention provides a method for making 3-HP that
20 uses a β -alanine intermediate. This method can be performed by contacting β -alanine CoA with a first polypeptide having β -alanyl-CoA ammonia lyase activity (such as a polypeptide having the amino acid sequence set forth in SEQ ID NO: 160 or 161) to form acrylyl-CoA, contacting acrylyl-CoA with a second polypeptide having 3-HP-CoA dehydratase activity to form 3-HP-CoA, and contacting 3-HP-CoA with a third
25 polypeptide having glutamate dehydrogenase activity to make 3-HP.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable
30 methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In

case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

5

DESCRIPTION OF DRAWINGS

Figure 1 is a diagram of a pathway for making 3-HP.

Figure 2 is a diagram of a pathway for making polymerized 3-HP.

Figure 3 is a diagram of a pathway for making esters of 3-HP.

10 Figure 4 is a diagram of a pathway for making polymerized acrylic acid.

Figure 5 is a diagram of a pathway for making esters of acrylate.

Figure 6 is a listing of a nucleic acid sequence that encodes a polypeptide having CoA transferase activity (SEQ ID NO:1).

15 Figure 7 is a listing of an amino acid sequence of a polypeptide having CoA transferase activity (SEQ ID NO:2).

Figure 8 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:1, 3, 4, and 5.

Figure 9 is an alignment of the amino acid sequences set forth in SEQ ID NOs:2, 6, 7, and 8.

20 Figure 10 is a listing of a nucleic acid sequence that encodes a polypeptide having E1 activator activity (SEQ ID NO:9).

Figure 11 is a listing of an amino acid sequence of a polypeptide having E1 activator activity (SEQ ID NO:10).

25 Figure 12 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:9, 11, 12, and 13.

Figure 13 is an alignment of the amino acid sequences set forth in SEQ ID NOs:10, 14, 15, and 16.

Figure 14 is a listing of a nucleic acid sequence that encodes an E2 α subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:17).

30 Figure 15 is a listing of an amino acid sequence of an E2 α subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:18).

Figure 16 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:17, 19, 20, and 21.

Figure 17 is an alignment of the amino acid sequences set forth in SEQ ID NOs:18, 22, 23, and 24.

5 Figure 18 is a listing of a nucleic acid sequence that encodes an E2 β subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:25). The "G" at position 443 can be an "A"; and the "A" at position 571 can be a "G".

Figure 19 is a listing of an amino acid sequence of an E2 β subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:26).

10 Figure 20 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:25, 27, 28, and 29.

Figure 21 is an alignment of the amino acid sequences set forth in SEQ ID NOs:26, 30, 31, and 32.

15 Figure 22 is a listing of a nucleic acid sequence of genomic DNA from *Megasphaera elsdenii* (SEQ ID NO:33):

Figure 23 is a listing of a nucleic acid sequence that encodes a polypeptide from *Megasphaera elsdenii* (SEQ ID NO:34).

Figure 24 is a listing of an amino acid sequence of a polypeptide from *Megasphaera elsdenii* (SEQ ID NO:35).

20 Figure 25 is a listing of a nucleic acid sequence that encodes a polypeptide having enzymatic activity (SEQ ID NO:36).

Figure 26 is a listing of an amino acid sequence of a polypeptide having enzymatic activity (SEQ ID NO:37).

25 Figure 27 is a listing of a nucleic acid sequence that contains non-coding as well as coding sequence of a polypeptide having CoA synthase, dehydratase, and dehydrogenase activity (SEQ ID NO:38). The start site for the coding sequence is at position 480, a ribosome binding site is at position 466-473, and the stop codon is at position 5946.

30 Figure 28 is a listing of an amino acid sequence from a polypeptide having CoA synthase, dehydratase, and dehydrogenase activity (SEQ ID NO:39).

Figure 29 is a listing of a nucleic acid sequence that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (SEQ ID NO:40).

Figure 30 is a listing of an amino acid sequence of a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (SEQ ID NO:41).

5 Figure 31 is a listing of a nucleic acid sequence that contains non-coding as well as coding sequence of a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (SEQ ID NO:42).

Figure 32 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:40, 43, 44, and 45.

10 Figure 33 is an alignment of the amino acid sequences set forth in SEQ ID NOs:41, 46, 47, and 48.

Figure 34 is a diagram of the construction of a synthetic operon (pTDH) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

15 Figure 35A and B is a diagram of the construction of a synthetic operon (pHTD) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

20 Figure 36A and B is a diagram of the construction of a synthetic operon (pEIITHrEI) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

Figure 37A and B is a diagram of the construction of a synthetic operon (pEIITHEI) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

25 Figure 38A and B is a diagram of the construction of two plasmids, pEIITH and pPROEI. The pEIITH plasmid encodes polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E2 α and E2 β), and 3-hydroxypropionyl-CoA

30

dehydratase activity (3-HP-CoA dehydratase), and the pPROEI plasmid encodes a polypeptide having E1 activator activity.

Figure 39 is a listing of a nucleic acid sequence that encodes a polypeptide having CoA synthase, dehydratase, and dehydrogenase activity (SEQ ID NO:129).

5 Figure 40 is an alignment of the amino acid sequences set forth in SEQ ID NOs:39, 130, and 131. The uppercase amino acid residues represent positions where that amino acid residue is present in two or more sequences.

Figure 41 is an alignment of the amino acid sequences set forth in SEQ ID NOs:39, 132, and 133. The uppercase amino acid residues represent positions where that amino acid residue is present in two or more sequences.

10

Figure 42 is an alignment of the amino acid sequences set forth in SEQ ID NOs: 39, 134, and 135. The uppercase amino acid residues represent positions where that amino acid residue is present in two or more sequences.

Figure 43 is a diagram of several pathways for making organic compounds using the multifunctional OS17 enzyme:

15

Figure 44 is a diagram of a pathway for making 3-HP via acetyl-CoA and malonyl-CoA.

Figure 45 is a diagram of pMSD8, pET30a/acc1, pFN476, and PET286 constructs.

Figure 46 contains a total ion chromatogram and five mass spectrums of Coenzyme-A thioesters. Panel A is total ion chromatogram illustrating the separation of Coenzyme A and four CoA-organic thioesters: 1=Coenzyme A, 2=lactyl-CoA, 3=acetyl-CoA, 4=acrylyl-CoA, 5=propionyl-CoA. Panel B is a mass spectrum of Coenzyme A. Panel C is a mass spectrum of lactyl-CoA. Panel D is a mass spectrum of acetyl-CoA. Panel E is a mass spectrum of acrylyl-CoA. Panel F is a mass spectrum of propionyl-CoA.

20

Figure 47 contains ion chromatograms and mass spectrums. Panel A is a total ion chromatogram of a mixture of lactyl-CoA and 3-HP-CoA. The Panel A insert is the mass spectrum recorded under peak 1. Panel B is a total ion chromatogram of lactyl-CoA. The Panel B insert is the mass spectrum recorded under peak 2. In each panel, peak 1 is 3-HP-CoA, and peak 2 is lactyl-CoA. The peak labeled with an asterisk was confirmed not to be a CoA ester.

25

30

Figure 48 contains ion chromatograms and mass spectrums. Panel A is a total ion chromatogram of CoA esters derived from a broth produced by *E. coli* transfected with pEIITHrEI. The Panel A insert is the mass spectrum recorded under peak 1. Panel B is a total ion chromatogram of CoA esters derived from a broth produced by control *E. coli* not transfected with pEIITHrEI. The Panel B insert is the mass spectrum recorded under peak 2. In each panel, peak 1 is 3-HP-CoA, and peak 2 is lactyl-CoA. The peaks labeled with an asterisk were confirmed not to be a CoA ester.

Figure 49 is a listing of a nucleic acid sequence that encodes a polypeptide having malonyl-CoA reductase activity (SEQ ID NO: 140).

10 Figure 50 is a listing of an amino acid sequence of a polypeptide having malonyl-CoA reductase activity (SEQ ID NO:141).

Figure 51 is a listing of a nucleic acid sequence that encodes a portion of a polypeptide having malonyl-CoA reductase activity (SEQ ID NO:142).

15 Figure 52 is an alignment of the amino acid sequences set forth in SEQ ID NOs: 141, 143, 144, 145, 146, and 147.

Figure 53 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs: 140, 148, 149, 150, 151, and 152.

Figure 54 is a diagram of a pathway for making 3-HP via a β -alanine intermediate.

Figure 55 is a diagram of a pathway for making 3-HP via a β -alanine intermediate.

20 Figure 56 is a listing of an amino acid sequence of a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:160).

Figure 57 is a listing of an amino acid sequence of a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:161).

25 Figure 58 is a listing of a nucleic acid sequence that encodes a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:162).

Figure 59 is a listing of a nucleic acid sequence that can encode a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:163).

DETAILED DESCRIPTION

I. Terms

Nucleic acid: The term "nucleic acid" as used herein encompasses both RNA and DNA including, without limitation, cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear.

Isolated: The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or

eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

It will be apparent to those of skill in the art that a nucleic acid existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be
5 considered an isolated nucleic acid.

Exogenous: The term "exogenous" as used herein with reference to nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Thus, non-naturally-occurring nucleic acid is considered to be
10 exogenous to a cell once introduced into the cell. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of person X is an exogenous nucleic acid with respect to a cell of person Y once that chromosome is introduced into Y's cell.

Hybridization: The term "hybridization" as used herein refers to a method of
15 testing for complementarity in the nucleotide sequence of two nucleic acid molecules, based on the ability of complementary single-stranded DNA and/or RNA to form a duplex molecule. Nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, any nucleic acid having some
homology to a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129,
20 140, 142, 162, or 163 can be used as a probe to identify a similar nucleic acid by hybridization under conditions of moderate to high stringency. Once identified, the nucleic acid then can be purified, sequenced, and analyzed to determine whether it is within the scope of the invention as described herein.

Hybridization can be done by Southern or Northern analysis to identify a DNA or
25 RNA sequence, respectively, that hybridizes to a probe. The probe can be labeled with a biotin, digoxigenin, an enzyme, or a radioisotope such as ^{32}P . The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art such as those described in sections
30 7.39-7.52 of Sambrook et al., (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY. Typically, a probe is at least about 20 nucleotides in

length. For example, a probe corresponding to a 20 nucleotide sequence set forth in SEQ ID NO: 1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, or 142 can be used to identify an identical or similar nucleic acid. In addition, probes longer or shorter than 20 nucleotides can be used.

5 The invention also provides isolated nucleic acid sequences that are at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000 bases in length) and hybridize, under hybridization conditions, to the sense or antisense strand of a nucleic acid having the sequence set forth in SEQ ID NO: 1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142,
10 162, or 163. The hybridization conditions can be moderately or highly stringent hybridization conditions.

For the purpose of this invention, moderately stringent hybridization conditions mean the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO₄ (pH 7.4), 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated
15 salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about 5x10⁷ cpm/µg), while the washes are performed at about 50°C with a wash solution containing 2X SSC and 0.1% sodium dodecyl sulfate.

Highly stringent hybridization conditions mean the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO₄ (pH 7.4), 5X SSC, 5X
20 Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about 5x10⁷ cpm/µg), while the washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% sodium dodecyl sulfate.

Purified: The term "purified" as used herein does not require absolute purity;
25 rather, it is intended as a relative term. Thus, for example, a purified polypeptide or nucleic acid preparation can be one in which the subject polypeptide or nucleic acid, respectively, is at a higher concentration than the polypeptide or nucleic acid would be in its natural environment within an organism. For example, a polypeptide preparation can be considered purified if the polypeptide content in the preparation represents at least
30 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 98%, or 99% of the total protein content of the preparation.

Transformed: A "transformed" cell is a cell into which a nucleic acid molecule has been introduced by, for example, molecular biology techniques. As used herein, the term "transformation" encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell including, without limitation, transfection with a viral vector, conjugation, transformation with a plasmid vector, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Recombinant: A "recombinant" nucleic acid is one having (1) a sequence that is not naturally occurring in the organism in which it is expressed or (2) a sequence made by an artificial combination of two otherwise-separated, shorter sequences. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. "Recombinant" is also used to describe nucleic acid molecules that have been artificially manipulated, but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated.

Specific binding agent: A "specific binding agent" is an agent that is capable of specifically binding to any of the polypeptide described herein, and can include polyclonal antibodies, monoclonal antibodies (including humanized monoclonal antibodies), and fragments of monoclonal antibodies such as Fab, F(ab')₂, and Fv fragments as well as any other agent capable of specifically binding to an epitope of such polypeptides.

Antibodies to the polypeptides provided herein (or fragments thereof) can be used to purify or identify such polypeptides. The amino acid and nucleic acid sequences provided herein allow for the production of specific antibody-based binding agents that recognize the polypeptides described herein.

Monoclonal or polyclonal antibodies can be produced to the polypeptides, portions of the polypeptides, or variants thereof. Optimally, antibodies raised against one or more epitopes on a polypeptide antigen will specifically detect that polypeptide. That is, antibodies raised against one particular polypeptide would recognize and bind that particular polypeptide, and would not substantially recognize or bind to other polypeptides. The determination that an antibody specifically binds to a particular polypeptide is made by any one of a number of standard immunoassay methods; for

instance, Western blotting (See, e.g., Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

To determine that a given antibody preparation (such as a preparation produced in a mouse against a polypeptide having the amino acid sequence set forth in SEQ ID NO: 2) specifically detects the appropriate polypeptide (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO: 2) by Western blotting, total cellular protein can be extracted from cells and separated by SDS-polyacrylamide gel electrophoresis. The separated total cellular protein can then be transferred to a membrane (e.g., nitrocellulose), and the antibody preparation incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies can be detected using an appropriate secondary antibody (e.g., an anti-mouse antibody) conjugated to an enzyme such as alkaline phosphatase since application of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a densely blue-colored compound by immuno-localized alkaline phosphatase.

Substantially pure polypeptides suitable for use as an immunogen can be obtained from transfected cells, transformed cells, or wild-type cells. Polypeptide concentrations in the final preparation can be adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. In addition, polypeptides ranging in size from full-length polypeptides to polypeptides having as few as nine amino acid residues can be utilized as immunogens. Such polypeptides can be produced in cell culture, can be chemically synthesized using standard methods, or can be obtained by cleaving large polypeptides into smaller polypeptides that can be purified. Polypeptides having as few as nine amino acid residues in length can be immunogenic when presented to an immune system in the context of a Major Histocompatibility Complex (MHC) molecule such as an MHC class I or MHC class II molecule. Accordingly, polypeptides having at least 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 900, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, or more consecutive amino acid residues of any

amino acid sequence disclosed herein can be used as immunogens for producing antibodies.

Monoclonal antibodies to any of the polypeptides disclosed herein can be prepared from murine hybridomas according to the classic method of Kohler & Milstein (Nature 256:495 (1975)) or a derivative method thereof.

Polyclonal antiserum containing antibodies to the heterogeneous epitopes of any polypeptide disclosed herein can be prepared by immunizing suitable animals with the polypeptide (or fragment thereof), which can be unmodified or modified to enhance immunogenicity. An effective immunization protocol for rabbits can be found in Vaitukaitis *et al.* (*J. Clin. Endocrinol. Metab.* 33:988-991 (1971)).

Antibody fragments can be used in place of whole antibodies and can be readily expressed in prokaryotic host cells. Methods of making and using immunologically effective portions of monoclonal antibodies, also referred to as "antibody fragments," are well known and include those described in Better & Horowitz (*Methods Enzymol.* 178:476-496 (1989)), Glockshuber *et al.* (*Biochemistry* 29:1362-1367 (1990), U.S. Pat. No. 5,648,237 ("Expression of Functional Antibody Fragments"), U.S. Pat. No. 4,946,778 ("Single Polypeptide Chain Binding Molecules"), U.S. Pat. No. 5,455,030 ("Immunotherapy Using Single Chain Polypeptide Binding Molecules"), and references cited therein.

Operably linked: A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence whenever the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two polypeptide-coding regions, in the same reading frame.

Probes and primers: Nucleic acid probes and primers can be prepared readily based on the amino acid sequences and nucleic acid sequences provided herein. A "probe" includes an isolated nucleic acid containing a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed in, for example, Sambrook *et al.* (ed.), *Molecular Cloning*:

A Laboratory Manual 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, and Ausubel *et al.* (ed.) Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987.

- 5 “Primers” are typically nucleic acid molecules having ten or more nucleotides (e.g., nucleic acid molecules having between about 10 nucleotides and about 100 nucleotides). A primer can be annealed to a complementary target nucleic acid strand by nucleic acid hybridization to form a hybrid between the primer and the target nucleic acid strand, and then extended along the target nucleic acid strand by, for example, a DNA
10 polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, for example, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

- Methods for preparing and using probes and primers are described, for example, in references such as Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual,
15 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; Ausubel *et al.* (ed.), Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987; and Innis *et al.*, PCR
Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR
20 primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, .COPYRG. 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with the length, but that a probe or primer can range in size from a full-length sequence to sequences as short as five consecutive nucleotides. Thus, for example, a primer of 20
25 consecutive nucleotides can anneal to a target with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers can be selected that comprise, for example, 10, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550,
30 1600, 1650, 1700, 1750, 1800, 1850, 1900, 2000, 2050, 2100, 2150, 2200, 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2600, 2650, 2700, 2750, 2800, 2850, 2900, 3000, 3050,

3100, 3150, 3200, 3250, 3300, 3350, 3400, 3450, 3500, 3550, 3600, 3650, 3700, 3750, 3800, 3850, 3900, 4000, 4050, 4100, 4150, 4200, 4250, 4300, 4350, 4400, 4450, 4500, 4550, 4600, 4650, 4700, 4750, 4800, 4850, 4900, 5000, 5050, 5100, 5150, 5200, 5250, 5300, 5350, 5400, 5450, or more consecutive nucleotides.

5 **Percent sequence identity:** The "percent sequence identity" between a particular nucleic acid or amino acid sequence and a sequence referenced by a particular sequence identification number is determined as follows. First, a nucleic acid or amino acid sequence is compared to the sequence set forth in a particular sequence identification number using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained from Fish & Richardson's web site (www.fr.com) or the United States government's National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to -1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the

designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence (e.g., SEQ ID NO:1), or by an articulated length (e.g., 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with the sequence set forth in SEQ ID NO:1 is 75.0 percent identical to the sequence set forth in SEQ ID NO:1 (i.e., $1166 \div 1554 \times 100 = 75.0$). It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 is rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 is rounded up to 75.2. It is also noted that the length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (i.e., $15 \div 20 \times 100 = 75$).

	1	20
Target Sequence:	AGGTCGTGTACTGTCAGTCA	
Identified Sequence:	ACGTGGTGAAGTCCAGTGA	

Conservative substitution: The term "conservative substitution" as used herein refers to any of the amino acid substitutions set forth in Table 1. Typically, conservative substitutions have little to no impact on the activity of a polypeptide. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR.

Table 1

Original Residue	Conservative Substitution(s)
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

II. Metabolic Pathways

The invention provides methods and materials related to producing 3-HP as well as other organic compounds (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP). Specifically, the invention provides isolated nucleic acids, polypeptides, host cells, and methods and materials for producing 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP.

Accordingly, the invention provides several metabolic pathways that can be used to produce organic compounds from PEP (Figures 1-5, 43-44, 54, and 55). As depicted in Figure 1, lactate can be converted into lactyl-CoA by a polypeptide having CoA transferase activity (EC 2.8.3.1); the resulting lactyl-CoA can be converted into acrylyl-CoA by a polypeptide (or multiple polypeptide complex such as an activated E2 α and E2 β complex) having lactyl-CoA dehydratase activity (EC 4.2.1.54); the resulting acrylyl-CoA can be converted into 3-hydroxypropionyl-CoA (3-HP-CoA) by a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (EC 4.2.1.-); and the resulting 3-HP-CoA can be converted into 3-HP by a polypeptide having CoA transferase activity, a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), or a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity (EC 3.1.2.4).

Polypeptides having CoA transferase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Megasphaera elsdenii*, *Clostridium propionicum*, *Clostridium kluyveri*, and *Escherichia coli*. For example, nucleic acid that encodes a polypeptide having CoA transferase activity can be obtained from *Megasphaera elsdenii* as described in Example 1 and can have a sequence as set forth in SEQ ID NO: 1. In addition, polypeptides having CoA transferase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 1 provided herein can be used to encode a polypeptide having CoA transferase activity.

Polypeptides (or the polypeptides of a multiple polypeptide complex such as an activated E2 α and E2 β complex) having lactyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Megasphaera elsdenii* and *Clostridium propionicum*. For example, nucleic acid encoding an E1 activator, an E2 α subunit, and an E2 β subunit that can form a multiple polypeptide complex having lactyl-CoA dehydratase activity can be obtained from *Megasphaera elsdenii* as described in Example 2. The nucleic acid encoding the E1 activator can contain a sequence as set forth in SEQ ID NO: 9; the nucleic acid encoding the E2 α subunit can contain a sequence as set forth in SEQ ID NO: 17; and the nucleic acid encoding the E2 β subunit can contain a sequence as set forth in SEQ ID NO: 25. In addition, polypeptides (or the polypeptides of a multiple polypeptide complex) having

lactyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 9, 17, and 25 provided herein can be used to encode the polypeptides of a multiple polypeptide complex having CoA transferase activity.

5 Polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Chloroflexus aurantiacus*, *Candida rugosa*, *Rhodospirillum rubrum*, and *Rhodobacter capsulatus*. For example, nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be obtained from *Chloroflexus*
10 *aurantiacus* as described in Example 3 and can have a sequence as set forth in SEQ ID NO: 40. In addition, polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 40 provided herein can be used to encode a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity.

15 Polypeptides having 3-hydroxypropionyl-CoA hydrolase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Candida rugosa*. Polypeptides having 3-hydroxyisobutryl-CoA hydrolase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Pseudomonas fluorescens*, *rattus*, and
20 *homo sapiens*. For example, nucleic acid that encodes a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity can be obtained from *homo sapiens* and can have a sequence as set forth in GenBank® accession number U66669.

The term "polypeptide having enzymatic activity" as used herein refers to any polypeptide that catalyzes a chemical reaction of other substances without itself being
25 destroyed or altered upon completion of the reaction. Typically, a polypeptide having enzymatic activity catalyzes the formation of one or more products from one or more substrates. Such polypeptides can have any type of enzymatic activity including, without limitation, the enzymatic activity or enzymatic activities associated with enzymes such as dehydratases/hydratases, 3-hydroxypropionyl-CoA dehydratases/hydratases, CoA
30 transferases, lactyl-CoA dehydratases, 3-hydroxypropionyl-CoA hydrolases, 3-

hydroxyisobutryl-CoA hydrolases, poly hydroxyacid synthases, CoA synthetases, malonyl-CoA reductases, β -alanine ammonia lyases, and lipases.

As depicted in Figure 2, lactate can be converted into lactyl-CoA by a polypeptide having CoA synthetase activity (EC 6.2.1.-); the resulting lactyl-CoA can be converted
5 into acrylyl-CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can be converted into 3-HP-CoA by a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity; and the resulting 3-HP-CoA can be converted into polymerized 3-HP by a polypeptide having poly hydroxyacid synthase activity (EC 2.3.1.-). Polypeptides having CoA synthetase activity
10 as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Escherichia coli*, *Rhodobacter sphaeroides*, *Saccharomyces cerevisiae*, and *Salmonella enterica*. For example, nucleic acid that encodes a polypeptide having CoA synthetase activity can be obtained from *Escherichia coli* and can have a sequence as set forth in GenBank® accession number U00006. Polypeptides (or multiple
15 polypeptide complexes) having lactyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained as provided herein. Polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides also can be obtained as provided herein. Polypeptides having poly hydroxyacid synthase activity as well as nucleic acid encoding such polypeptides can be
20 obtained from various species including, without limitation, *Rhodobacter sphaeroides*, *Comamonas acidovorans*, *Ralstonia eutropha*, and *Pseudomonas oleovorans*. For example, nucleic acid that encodes a polypeptide having poly hydroxyacid synthase activity can be obtained from *Rhodobacter sphaeroides* and can have a sequence as set forth in GenBank® accession number X97200.

25 As depicted in Figure 3, lactate can be converted into lactyl-CoA by a polypeptide having CoA transferase activity; the resulting lactyl-CoA can be converted into acrylyl-CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can be converted into 3-HP-CoA by a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity; the resulting 3-HP-CoA can be
30 converted into 3-HP by a polypeptide having CoA transferase activity, a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity, or a polypeptide having 3-

hydroxyisobutryl-CoA hydrolase activity; and the resulting 3-HP can be converted into an ester of 3-HP by a polypeptide having lipase activity (EC 3.1.1.-). Polypeptides having lipase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Candida rugosa*, *Candida tropicalis*, and
5 *Candida albicans*. For example, nucleic acid that encodes a polypeptide having lipase activity can be obtained from *Candida rugosa* and can have a sequence as set forth in GenBank® accession number A81171.

As depicted in Figure 4, lactate can be converted into lactyl-CoA by a polypeptide having CoA synthetase activity; the resulting lactyl-CoA can be converted into acrylyl-CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase
10 activity; and the resulting acrylyl-CoA can be converted into polymerized acrylate by a polypeptide having poly hydroxyacid synthase activity.

As depicted in Figure 5, lactate can be converted into lactyl-CoA by a polypeptide having CoA transferase activity; the resulting lactyl-CoA can be converted into acrylyl-CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase
15 activity; the resulting acrylyl-CoA can be converted into acrylate by a polypeptide having CoA transferase activity; and the resulting acrylate can be converted into an ester of acrylate by a polypeptide having lipase activity.

As depicted in Figure 44, acetyl-CoA can be converted into malonyl-CoA by a
20 polypeptide having acetyl-CoA carboxylase activity, and the resulting malonyl-CoA can be converted into 3-HP by a polypeptide having malonyl-CoA reductase activity. Polypeptides having acetyl-CoA carboxylase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Escherichia coli* and *Chloroflexus aurantiacus*. For example, nucleic acid that encodes a
25 polypeptide having acetyl-CoA carboxylase activity can be obtained from *Escherichia coli* and can have a sequence as set forth in GenBank® accession number M96394 or U18997. Polypeptides having malonyl-CoA reductase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Chloroflexus aurantiacus*, *Sulfolobus metacillus*, and *Acidiamus brierleyi*. For
30 example, nucleic acid that encodes a polypeptide having malonyl-CoA reductase activity can be obtained as described herein and can have a sequence similar to the sequence set

forth in SEQ ID NO: 140. In addition, polypeptides having malonyl-CoA reductase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 140 provided herein can be used to encode a polypeptide having malonyl-CoA reductase activity.

5 Polypeptides having malonyl-CoA reductase activity can use NADPH as a co-factor. For example, the polypeptide having the amino acid sequence set forth in SEQ ID NO: 141 is a polypeptide having malonyl-CoA reductase activity that uses NADPH as a co-factor when converting malonyl-CoA into 3-HP. Likewise, polypeptides having malonyl-CoA reductase activity can use NADH as a co-factor. Such polypeptides can be
10 obtained by converting a polypeptide that has malonyl-CoA reductase activity and uses NADPH as a cofactor into a polypeptide that has malonyl-CoA reductase activity and uses NADH as a cofactor. Any method can be used to convert a polypeptide that uses NADPH as a cofactor into a polypeptide that uses NADH as a cofactor such as those described by others (Eppink *et al.*, *J. Mol. Biol.*, 292(1):87-96 (1999), Hall and Tomsett,
15 *Microbiology*, 146(Pt 6):1399-406 (2000), and Dohr *et al.*, *Proc. Natl. Acad. Sci.*, 98(1):81-86 (2001)). For example, mutagenesis can be used to convert the polypeptide encoded by the nucleic acid sequence set forth in SEQ ID NO: 140 into a polypeptide that, when converting malonyl-CoA into 3-HP, uses NADH as a co-factor instead of NADPH.

20 As depicted in Figure 43, propionate can be converted into propionyl-CoA by a polypeptide having CoA synthetase activity such as the polypeptide having the sequence set forth in SEQ ID NO: 39; the resulting propionyl-CoA can be converted into acrylyl-CoA by a polypeptide having dehydrogenase activity such as the polypeptide having the sequence set forth in SEQ ID NO: 39; and the resulting acrylyl-CoA can be converted
25 into (1) acrylate by a polypeptide having CoA transferase activity or CoA hydrolase activity, (2) 3-HP-CoA by a polypeptide having 3-HP dehydratase activity (also referred to as acrylyl-CoA hydratase or simply hydratase) such as the polypeptide having the sequence set forth in SEQ ID NO:39, or (3) polymerized acrylate by a polypeptide having poly hydroxyacid synthase activity. The resulting acrylate can be converted into an ester
30 of acrylate by a polypeptide having lipase activity. The resulting 3-HP-CoA can be converted into (1) 3-HP by a polypeptide having CoA transferase activity, a polypeptide

having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), or a polypeptide having 3-hydroxyisobutyryl-CoA hydrolase activity (EC 3.1.2.4), or (2) polymerized 3-HP by a polypeptide having poly hydroxyacid synthase activity (EC 2.3.1.-).

As depicted in Figure 54, PEP can be converted into β -alanine. β -alanine can be converted into β -alanyl-CoA through the use of a polypeptide having CoA transferase activity. β -alanyl-CoA can then be converted into acrylyl-CoA through the use of a polypeptide having β -alanyl-CoA ammonia lyase activity. Acrylyl-CoA can then be converted into 3-HP-CoA through the use of a polypeptide having 3-HP-CoA dehydratase activity, and a polypeptide having glutamate dehydrogenase activity can be used to convert 3-HP-CoA into 3-HP.

As depicted in Figure 55, 3-HP can be made from β -alanine by first contacting β -alanine with a polypeptide having 4,4-aminobutyrate aminotransferase activity to create malonate semialdehyde. The malonate semialdehyde can be converted into 3-HP with a polypeptide having 3-HP dehydrogenase activity or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

III. Nucleic acid molecules and polypeptides

The invention provides isolated nucleic acid that contains the entire nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. In addition, the invention provides isolated nucleic acid that contains a portion of the nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. For example, the invention provides isolated nucleic acid that contains a 15 nucleotide sequence identical to any 15 nucleotide sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163 including, without limitation, the sequence starting at nucleotide number 1 and ending at nucleotide number 15, the sequence starting at nucleotide number 2 and ending at nucleotide number 16, the sequence starting at nucleotide number 3 and ending at nucleotide number 17, and so forth. It will be appreciated that the invention also provides isolated nucleic acid that contains a nucleotide sequence that is greater than 15 nucleotides (e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more nucleotides) in length and identical to any portion of the sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129,

140, 142, 162, or 163. For example, the invention provides isolated nucleic acid that contains a 25 nucleotide sequence identical to any 25 nucleotide sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163 including, without limitation, the sequence starting at nucleotide number 1 and ending at nucleotide number 25, the sequence starting at nucleotide number 2 and ending at nucleotide number 26, the sequence starting at nucleotide number 3 and ending at nucleotide number 27, and so forth. Additional examples include, without limitation, isolated nucleic acids that contain a nucleotide sequence that is 50 or more nucleotides (e.g., 100, 150, 200, 250, 300, or more nucleotides) in length and identical to any portion of the sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. Such isolated nucleic acids can include, without limitation, those isolated nucleic acids containing a nucleic acid sequence represented in a single line of sequence depicted in Figure 6, 10, 14, 18, 22, 23, 25, 27, 29, 31, 39, 49, or 51 since each line of sequence depicted in these figures, with the possible exception of the last line, provides a nucleotide sequence containing at least 50 bases.

In addition, the invention provides isolated nucleic acid that contains a variation of the nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. For example, the invention provides isolated nucleic acid containing a nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163 that contains a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such isolated nucleic acid molecules can share at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163.

The invention provides multiple examples of isolated nucleic acid that contains a variation of a nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. For example, Figure 8 provides the sequence set forth in SEQ ID NO:1 aligned with three other nucleic acid sequences. Examples of variations of the sequence set forth in SEQ ID NO:1 include, without limitation, any variation of the sequence set forth in SEQ ID NO:1 provided in Figure 8. Such variations are provided in

Figure 8 in that a comparison of the nucleotide (or lack thereof) at a particular position of the sequence set forth in SEQ ID NO:1 with the nucleotide (or lack thereof) at the same aligned position of any of the other three nucleic acid sequences depicted in Figure 8 (i.e., SEQ ID NOs:3, 4, and 5) provides a list of specific changes for the sequence set forth in SEQ ID NO:1. For example, the "a" at position 49 of SEQ ID NO:1 can be substituted with an "c" as indicated in Figure 8. As also indicated in Figure 8, the "a" at position 590 of SEQ ID NO:1 can be substituted with a "atgg"; an "aaac" can be inserted before the "g" at position 393 of SEQ ID NO:1; or the "gaa" at position 736 of SEQ ID NO:1 can be deleted. It will be appreciated that the sequence set forth in SEQ ID NO:1 can contain any number of variations as well as any combination of types of variations. For example, the sequence set forth in SEQ ID NO:1 can contain one variation provided in Figure 8 or more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, or more) of the variations provided in Figure 8. It is noted that the nucleic acid sequences provided by Figure 8 can encode polypeptides having CoA transferase activity. The invention also provides isolated nucleic acid that contains a variant of a portion of the sequence set forth in SEQ ID NO:1 as depicted in Figure 8 and described herein.

Likewise, Figure 12 provides variations of SEQ ID NO:9 and portions thereof; Figure 16 provides variations of SEQ ID NO:17 and portions thereof; Figure 20 provides variations of SEQ ID NO:25 and portions thereof; Figure 32 provides variations of SEQ ID NO:40 and portions thereof; and Figure 53 provides variations of SEQ ID NO:140.

The invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes the entire amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. In addition, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes a portion of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes a 15 amino acid sequence identical to any 15 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 15, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 16, the sequence starting at amino acid residue number 3 and ending

at amino acid residue number 17, and so forth. It will be appreciated that the invention also provides isolated nucleic acid that contains a nucleic acid sequence that encodes an amino acid sequence that is greater than 15 amino acid residues (e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes a 25 amino acid sequence identical to any 25 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 25, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 26, the sequence starting at amino acid residue number 3 and ending at amino acid residue number 27, and so forth. Additional examples include, without limitation, isolated nucleic acids that contain a nucleic acid sequence that encodes an amino acid sequence that is 50 or more amino acid residues (e.g., 100, 150, 200, 250, 300, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. Such isolated nucleic acids can include, without limitation, those isolated nucleic acids containing a nucleic acid sequence that encodes an amino acid sequence represented in a single line of sequence depicted in Figure 7, 11, 15, 19, 24, 26, 28, 30, or 50 since each line of sequence depicted in these figures, with the possible exception of the last line, provides an amino acid sequence containing at least 50 residues.

In addition, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes an amino acid sequence having a variation of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides isolated nucleic acid containing a nucleic acid sequence encoding an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 that contains a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such isolated nucleic acid molecules can contain a nucleic acid sequence encoding an amino acid sequence that shares at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99 percent sequence identity with a

sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161.

The invention provides multiple examples of isolated nucleic acid containing a nucleic acid sequence encoding an amino acid sequence having a variation of an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, Figure 9 provides the amino acid sequence set forth in SEQ ID NO:2 aligned with three other amino acid sequences. Examples of variations of the sequence set forth in SEQ ID NO:2 include, without limitation, any variation of the sequence set forth in SEQ ID NO:2 provided in Figure 9. Such variations are provided in Figure 9 in that a comparison of the amino acid residue (or lack thereof) at a particular position of the sequence set forth in SEQ ID NO:2 with the amino acid residue (or lack thereof) at the same aligned position of any of the other three amino acid sequences of Figure 9 (i.e., SEQ ID NOs:6, 7, and 8) provides a list of specific changes for the sequence set forth in SEQ ID NO:2. For example, the "k" at position 17 of SEQ ID NO:2 can be substituted with a "p" or "h" as indicated in Figure 9. As also indicated in Figure 9, the "v" at position 125 of SEQ ID NO:2 can be substituted with an "i" or "f". It will be appreciated that the sequence set forth in SEQ ID NO:2 can contain any number of variations as well as any combination of types of variations. For example, the sequence set forth in SEQ ID NO:2 can contain one variation provided in Figure 9 or more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, or more) of the variations provided in Figure 9. It is noted that the amino acid sequences provided in Figure 9 can be polypeptides having CoA transferase activity.

The invention also provides isolated nucleic acid containing a nucleic acid sequence encoding an amino acid sequence that contains a variant of a portion of the sequence set forth in SEQ ID NO:2 as depicted in Figure 9 and described herein.

Likewise, Figure 13 provides variations of SEQ ID NO:10 and portions thereof; Figure 17 provides variations of SEQ ID NO:18 and portions thereof; Figure 21 provides variations of SEQ ID NO:26 and portions thereof; Figure 33 provides variations of SEQ ID NO:41 and portions thereof; Figures 40, 41, and 42 provide variations of SEQ ID NO:39; and Figure 52 provides variations of SEQ ID NO:141 and portions thereof.

It is noted that codon preferences and codon usage tables for a particular species can be used to engineer isolated nucleic acid molecules that take advantage of the codon

usage preferences of that particular species. For example, the isolated nucleic acid provided herein can be designed to have codons that are preferentially used by a particular organism of interest.

The invention also provides isolated nucleic acid that is at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000 bases in length) and hybridizes, under hybridization conditions, to the sense or antisense strand of a nucleic acid having the sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. The hybridization conditions can be moderately or highly stringent hybridization conditions.

The invention provides polypeptides that contain the entire amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. In addition, the invention provides polypeptides that contain a portion of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides polypeptides that contain a 15 amino acid sequence identical to any 15 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 15, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 16, the sequence starting at amino acid residue number 3 and ending at amino acid residue number 17, and so forth. It will be appreciated that the invention also provides polypeptides that contain an amino acid sequence that is greater than 15 amino acid residues (e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides polypeptides that contain a 25 amino acid sequence identical to any 25 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 25, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 26, the sequence starting at amino acid residue number 3 and ending at amino acid residue number 27, and so forth. Additional examples include, without limitation, polypeptides

that contain an amino acid sequence that is 50 or more amino acid residues (e.g., 100, 150, 200, 250, 300, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. Such polypeptides can include, without limitation, those polypeptides containing a amino acid
5 sequence represented in a single line of sequence depicted in Figure 7, 11, 15, 19, 24, 26, 28, 30, or 50 since each line of sequence depicted in these figures, with the possible exception of the last line, provides an amino acid sequence containing at least 50 residues.

In addition, the invention provides polypeptides that an amino acid sequence having a variation of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35,
10 37, 39, 41, 141, 160, or 161. For example, the invention provides polypeptides containing an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 that contains a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such polypeptides can contain an
15 amino acid sequence that shares at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99 percent sequence identity with a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161.

The invention provides multiple examples of polypeptides containing an amino acid sequence having a variation of an amino acid sequence set forth in SEQ ID NO:2,
20 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, Figure 9 provides the amino acid sequence set forth in SEQ ID NO:2 aligned with three other amino acid sequences. Examples of variations of the sequence set forth in SEQ ID NO:2 include, without limitation, any variation of the sequence set forth in SEQ ID NO:2 provided in Figure 9. Such variations are provided in Figure 9 in that a comparison of the amino acid residue
25 (or lack thereof) at a particular position of the sequence set forth in SEQ ID NO:2 with the amino acid residue (or lack thereof) at the same aligned position of any of the other three amino acid sequences of Figure 9 (i.e., SEQ ID NOs:6, 7, and 8) provides a list of specific changes for the sequence set forth in SEQ ID NO:2. For example, the "k" at position 17 of SEQ ID NO:2 can be substituted with a "p" or "h" as indicated in Figure 9.
30 As also indicated in Figure 9, the "v" at position 125 of SEQ ID NO:2 can be substituted with an "i" or "f". It will be appreciated that the sequence set forth in SEQ ID NO:2 can

contain any number of variations as well as any combination of types of variations. For example, the sequence set forth in SEQ ID NO:2 can contain one variation provided in Figure 9 or more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, or more) of the variations provided in Figure 9. It is noted that the amino acid sequences provided in
5 Figure 9 can be polypeptides having CoA transferase activity.

The invention also provides polypeptides containing an amino acid sequence that contains a variant of a portion of the sequence set forth in SEQ ID NO:2 as depicted in Figure 9 and described herein.

Likewise, Figure 13 provides variations of SEQ ID NO:10 and portions thereof;
10 Figure 17 provides variations of SEQ ID NO:18 and portions thereof; Figure 21 provides variations of SEQ ID NO:26 and portions thereof; Figure 33 provides variations of SEQ ID NO:41 and portions thereof, Figures 40, 41, and 42 provide variations of SEQ ID NO:39; and Figure 52 provides variations of SEQ ID NO:141 and portions thereof.

Polypeptides having a variant amino acid sequence can retain enzymatic activity.
15 Such polypeptides can be produced by manipulating the nucleotide sequence encoding a polypeptide using standard procedures such as site-directed mutagenesis or PCR. One type of modification includes the substitution of one or more amino acid residues for amino acid residues having a similar biochemical property. For example, a polypeptide can have an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41,
20 141, 160, or 161 with one or more conservative substitutions.

More substantial changes can be obtained by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining: (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation; (b) the charge or
25 hydrophobicity of the polypeptide at the target site; or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in polypeptide function are those in which: (a) a hydrophilic residue, e.g., serine or threonine, is substituted for (or by) a hydrophobic residue, e.g., leucine, isoleucine, phenylalanine, valine or alanine; (b) a cysteine or proline is substituted for (or by) any other residue; (c)
30 a residue having an electropositive side chain, e.g., lysine, arginine, or histidine, is substituted for (or by) an electronegative residue, e.g., glutamic acid or aspartic acid; or

(d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. The effects of these amino acid substitutions (or other deletions or additions) can be assessed for polypeptides having enzymatic activity by analyzing the ability of the polypeptide to catalyze the conversion of the same
5 substrate as the related native polypeptide to the same product as the related native polypeptide. Accordingly, polypeptides having 5, 10, 20, 30, 40, 50 or less conservative substitutions are provided by the invention.

Polypeptides and nucleic acid encoding polypeptide can be produced by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these
10 techniques are provided in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual* 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring, Harbor, N.Y., 1989, Ch. 15. Nucleic acid molecules can contain changes of a coding region to fit the codon usage bias of the particular organism into which the molecule is to be introduced.

15 Alternatively, the coding region can be altered by taking advantage of the degeneracy of the genetic code to alter the coding sequence in such a way that, while the nucleic acid sequence is substantially altered, it nevertheless encodes a polypeptide having an amino acid sequence identical or substantially similar to the native amino acid sequence. For example, the ninth amino acid residue of the sequence set forth in SEQ ID
20 NO: 2 is alanine, which is encoded in the open reading frame by the nucleotide codon triplet GCT. Because of the degeneracy of the genetic code, three other nucleotide codon triplets—GCA, GCC, and GCG --also code for alanine. Thus, the nucleic acid sequence of the open reading frame can be changed at this position to any of these three codons without affecting the amino acid sequence of the encoded polypeptide or the
25 characteristics of the polypeptide. Based upon the degeneracy of the genetic code, nucleic acid variants can be derived from a nucleic acid sequence disclosed herein using a standard DNA mutagenesis techniques as described herein, or by synthesis of nucleic acid sequences. Thus, this invention also encompasses nucleic acid molecules that encode the same polypeptide but vary in nucleic acid sequence by virtue of the degeneracy of the
30 genetic code.

IV. Methods of Making 3-HP and Other Organic Acids

Each step provided in the pathways depicted in Figures 1-5, 43-44, 54, and 55 can be performed within a cell (*in vivo*) or outside a cell (*in vitro*, e.g., in a container or column). Additionally, the organic acid products can be generated through a combination of *in vivo* synthesis and *in vitro* synthesis. Moreover, the *in vitro* synthesis step, or steps, can be via chemical reaction or enzymatic reaction.

For example, a microorganism provided herein can be used to perform the steps provided in Figure 1, or an extract containing polypeptides having the indicated enzymatic activities can be used to perform the steps provided in Figure 1. In addition, chemical treatments can be used to perform the conversions provided in Figures 1-5, 43-44, 54, and 55. For example, acrylyl-CoA can be converted into acrylate by hydrolysis. Other chemical treatments include, without limitation, trans esterification to convert acrylate into an acrylate ester.

Carbon sources suitable as starting points for bioconversion include carbohydrates and synthetic intermediates. Examples of carbohydrates which cells are capable of metabolizing to pyruvate include sugars such as dextrose, triglycerides, and fatty acids.

Additionally, intermediate chemical products can be starting points. For example, acetic acid and carbon dioxide can be introduced into a fermentation broth. Acetyl-CoA, malonyl-CoA, and 3-HP can be sequentially produced using a polypeptide having CoA synthase activity, a polypeptide having acetyl-CoA carboxylase activity, and a polypeptide having malonyl-CoA reductase activity. Other useful intermediate chemical starting points can include propionic acid, acrylic acid, lactic acid, pyruvic acid, and β -alanine.

A. Expression of Polypeptides

The polypeptides described herein can be produced individually in a host cell or in combination in a host cell. Moreover, the polypeptides having a particular enzymatic activity can be a polypeptide that is either naturally-occurring or non-naturally-occurring. A naturally-occurring polypeptide is any polypeptide having an amino acid sequence as found in nature, including wild-type and polymorphic polypeptides. Such naturally-occurring polypeptides can be obtained from any species including, without limitation,

animal (e.g., mammalian), plant, fungal, and bacterial species. A non-naturally-occurring polypeptide is any polypeptide having an amino acid sequence that is not found in nature. Thus, a non-naturally-occurring polypeptide can be a mutated version of a naturally-occurring polypeptide, or an engineered polypeptide. For example, a non-naturally-occurring polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be a mutated version of a naturally-occurring polypeptide having 3-hydroxypropionyl-CoA dehydratase activity that retains at least some 3-hydroxypropionyl-CoA dehydratase activity. A polypeptide can be mutated by, for example, sequence additions, deletions, substitutions, or combinations thereof.

10 The invention provides genetically modified cells that can be used to perform one or more steps of the steps in the metabolic pathways described herein or the genetically modified cells can be used to produce the disclosed polypeptides for subsequent use *in vitro*. For example, an individual microorganism can contain exogenous nucleic acid such that each of the polypeptides necessary to perform the steps depicted in Figures 1, 2, 3, 4, 5, 43, 44, 54, or 55 are expressed. It is important to note that such cells can contain any number of exogenous nucleic acid molecules. For example, a particular cell can contain six exogenous nucleic acid molecules with each one encoding one of the six polypeptides necessary to convert lactate into 3-HP as depicted in Figure 1, or a particular cell can endogenously produce polypeptides necessary to convert lactate into acrylyl-CoA while containing exogenous nucleic acid that encodes polypeptides necessary to convert acrylyl-CoA into 3-HP.

20 In addition, a single exogenous nucleic acid molecule can encode one or more than one polypeptide. For example, a single exogenous nucleic acid molecule can contain sequences that encode three different polypeptides. Further, the cells described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid molecule. For example, a particular cell can contain about 50 copies of the constructs depicted in Figure 34, 35, 36, 37, 38, or 45. Again, the cells described herein can contain more than one particular exogenous nucleic acid molecule. For example, a particular cell can contain about 50 copies of exogenous nucleic acid molecule X as well as about 75 copies of exogenous nucleic acid molecule Y.

In another embodiment, a cell within the scope of the invention can contain an exogenous nucleic acid molecule that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. Such cells can have any level of 3-hydroxypropionyl-CoA dehydratase activity. For example, a cell containing an exogenous nucleic acid molecule that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can have 3-hydroxypropionyl-CoA dehydratase activity with a specific activity greater than about 1 mg 3-HP-CoA formed per gram dry cell weight per hour (e.g., greater than about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, or more mg 3-HP-CoA formed per gram dry cell weight per hour). Alternatively, a cell can have 3-hydroxypropionyl-CoA dehydratase activity such that a cell extract from 1×10^6 cells has a specific activity greater than about 1 μ g 3-HP-CoA formed per mg total protein per 10 minutes (e.g., greater than about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, or more μ g 3-HP-CoA formed per mg total protein per 10 minutes).

A nucleic acid molecule encoding a polypeptide having enzymatic activity can be identified and obtained using any method such as those described herein. For example, nucleic acid molecules that encode a polypeptide having enzymatic activity can be identified and obtained using common molecular cloning or chemical nucleic acid synthesis procedures and techniques, including PCR. In addition, standard nucleic acid sequencing techniques and software programs that translate nucleic acid sequences into amino acid sequences based on the genetic code can be used to determine whether or not a particular nucleic acid has any sequence homology with known enzymatic polypeptides. Sequence alignment software such as MEGALIGN[®] (DNASTAR, Madison, WI, 1997) can be used to compare various sequences. In addition, nucleic acid molecules encoding known enzymatic polypeptides can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and base substitutions, as well as combinations of deletions, insertions, and base substitutions. Further, nucleic acid and amino acid databases (e.g., GenBank[®]) can be used to identify a nucleic acid sequence that encodes a polypeptide having enzymatic activity. Briefly, any amino acid sequence having some homology to a polypeptide having enzymatic activity, or any nucleic acid sequence

having some homology to a sequence encoding a polypeptide having enzymatic activity can be used as a query to search GenBank®. The identified polypeptides then can be analyzed to determine whether or not they exhibit enzymatic activity.

In addition, nucleic acid hybridization techniques can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. Briefly, any nucleic acid molecule that encodes a known enzymatic polypeptide, or fragment thereof, can be used as a probe to identify a similar nucleic acid molecules by hybridization under conditions of moderate to high stringency. Such similar nucleic acid molecules then can be isolated, sequenced, and analyzed to determine whether the encoded polypeptide has enzymatic activity.

Expression cloning techniques also can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. For example, a substrate known to interact with a particular enzymatic polypeptide can be used to screen a phage display library containing that enzymatic polypeptide. Phage display libraries can be generated as described elsewhere (Burritt *et al.*, *Anal. Biochem.* 238:1-13 (1990)), or can be obtained from commercial suppliers such as Novagen (Madison, WI).

Further, polypeptide sequencing techniques can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. For example, a purified polypeptide can be separated by gel electrophoresis, and its amino acid sequence determined by, for example, amino acid microsequencing techniques. Once determined, the amino acid sequence can be used to design degenerate oligonucleotide primers. Degenerate oligonucleotide primers can be used to obtain the nucleic acid encoding the polypeptide by PCR. Once obtained, the nucleic acid can be sequenced, cloned into an appropriate expression vector, and introduced into a microorganism.

Any method can be used to introduce an exogenous nucleic acid molecule into a cell. In fact, many methods for introducing nucleic acid into microorganisms such as bacteria and yeast are well known to those skilled in the art. For example, heat shock, lipofection, electroporation, conjugation, fusion of protoplasts, and biolistic delivery are common methods for introducing nucleic acid into bacteria and yeast cells. See, e.g., Ito

et al., *J. Bacterol.* 153:163-168 (1983); Durrens *et al.*, *Curr. Genet.* 18:7-12 (1990); and Becker and Guarente, *Methods in Enzymology* 194:182-187 (1991).

An exogenous nucleic acid molecule contained within a particular cell of the invention can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state. In other words, a cell of the invention can be a stable or transient transformant. Again, a microorganism described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid molecule as described herein.

Methods for expressing an amino acid sequence from an exogenous nucleic acid molecule are well known to those skilled in the art. Such methods include, without limitation, constructing a nucleic acid such that a regulatory element promotes the expression of a nucleic acid sequence that encodes a polypeptide. Typically, regulatory elements are DNA sequences that regulate the expression of other DNA sequences at the level of transcription. Thus, regulatory elements include, without limitation, promoters, enhancers, and the like. Any type of promoter can be used to express an amino acid sequence from an exogenous nucleic acid molecule. Examples of promoters include, without limitation, constitutive promoters, tissue-specific promoters, and promoters responsive or unresponsive to a particular stimulus (e.g., light, oxygen, chemical concentration, and the like). Moreover, methods for expressing a polypeptide from an exogenous nucleic acid molecule in cells such as bacterial cells and yeast cells are well known to those skilled in the art. For example, nucleic acid constructs that are capable of expressing exogenous polypeptides within *E. coli* are well known. See, e.g., Sambrook *et al.*, *Molecular cloning: a laboratory manual*, Cold Spring Harbour Laboratory Press, New York, USA, second edition (1989).

B. Production of Organic Acids and Related Products via Host Cells

The nucleic acid and amino acid sequences provided herein can be used with cells to produce 3-HP and/or other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP. Such cells can be from any species including those listed within the taxonomy web pages at the

National Institute of Health sponsored by the United States government (www.ncbi.nlm.nih.gov). The cells can be eukaryotic or prokaryotic. For example, genetically modified cells of the invention can be mammalian cells (e.g., human, murine, and bovine cells), plant cells (e.g., corn, wheat, rice, and soybean cells), fungal cells (e.g., *Aspergillus* and *Rhizopus* cells), yeast cells, or bacterial cells (e.g., *Lactobacillus*, *Lactococcus*, *Bacillus*, *Escherichia*, and *Clostridium* cells). A cell of the invention also can be a microorganism. The term "microorganism" as used herein refers to any microscopic organism including, without limitation, bacteria, algae, fungi, and protozoa. Thus, *E. coli*, *S. cerevisiae*, *Kluyveromyces lactis*, *Candida blankii*, *Candida rugosa*, and *Pichia pastoris* are considered microorganisms and can be used as described herein.

Typically, a cell of the invention is genetically modified such that a particular organic compound is produced. In one embodiment, the invention provides cells that make 3-HP from PEP. Examples biosynthetic pathways that can be used by cells to make 3-HP are shown in Figures 1-5, 43-44, 54, and 55.

Generally, cells that are genetically modified to synthesize a particular organic compound contain one or more exogenous nucleic acid molecules that encode polypeptides having specific enzymatic activities. For example, a microorganism can contain exogenous nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. In this case, acrylyl-CoA can be converted into 3-hydroxypropionic acid-CoA which can lead to the production of 3-HP. It is noted that a cell can be given an exogenous nucleic acid molecule that encodes a polypeptide having an enzymatic activity that catalyzes the production of a compound not normally produced by that cell. Alternatively, a cell can be given an exogenous nucleic acid molecule that encodes a polypeptide having an enzymatic activity that catalyzes the production of a compound that is normally produced by that cell. In this case, the genetically modified cell can produce more of the compound, or can produce the compound more efficiently, than a similar cell not having the genetic modification.

In one embodiment, the invention provides a cell containing an exogenous nucleic acid molecule that encodes a polypeptide having enzymatic activity that leads to the formation of 3-HP. It is noted that the produced 3-HP can be secreted from the cell, eliminating the need to disrupt cell membranes to retrieve the organic compound.

Typically, the cell of the invention produces 3-HP with the concentration being at least about 100 mg per L (e.g., at least about 1 g/L, 5 g/L, 10 g/L, 25 g/L, 50 g/L, 75 g/L, 80 g/L, 90 g/L, 100 g/L, or 120 g/L). When determining the yield of an organic compound such as 3-HP for a particular cell, any method can be used. See, e.g., *Applied*

- 5 *Environmental Microbiology* 59(12):4261-4265 (1993). Typically, a cell within the scope of the invention such as a microorganism catabolizes a hexose carbon source such as glucose. A cell, however, can catabolize a variety of carbon sources such as pentose sugars (e.g., ribose, arabinose, xylose, and lyxose), fatty acids, acetate, or glycerols. In other words, a cell within the scope of the invention can utilize a variety of carbon
10 sources.

- As described herein, a cell within the scope of the invention can contain an exogenous nucleic acid molecule that encodes a polypeptide having enzymatic activity that leads to the formation of 3-HP or other organic compounds such as 1,3-propanediol, acrylic acid, poly-acrylate, acrylate-esters, 3-HP-esters, and poly-3-HP. Methods of
15 identifying cells that contain exogenous nucleic acid are well known to those skilled in the art. Such methods include, without limitation, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis (see hybridization described herein). In some cases, immunohisto-chemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of the
20 polypeptide encoded by that particular nucleic acid molecule. For example, an antibody having specificity for a polypeptide can be used to determine whether or not a particular cell contains nucleic acid encoding that polypeptide. Further, biochemical techniques can be used to determine if a cell contains a particular nucleic acid molecule encoding a polypeptide having enzymatic activity by detecting an organic product produced as a
25 result of the expression of the polypeptide having enzymatic activity. For example, detection of 3-HP after introduction of exogenous nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity into a cell that does not normally express such a polypeptide can indicate that that cell not only contains the introduced exogenous nucleic acid molecule but also expresses the encoded polypeptide from that
30 introduced exogenous nucleic acid molecule. Methods for detecting specific enzymatic activities or the presence of particular organic products are well known to those skilled in

the art. For example, the presence of an organic compound such as 3-HP can be determined as described elsewhere. See, Sullivan and Clarke, *J. Assoc. Offic. Agr. Chemists*, 38:514-518 (1955).

5 **C. Cells with Reduced Polypeptide Activity**

The invention also provides genetically modified cells having reduced polypeptide activity. The term "reduced" as used herein with respect to a cell and a particular polypeptide's activity refers to a lower level of activity than that measured in a comparable cell of the same species. For example, a particular microorganism lacking
10 enzymatic activity X is considered to have reduced enzymatic activity X if a comparable microorganism has at least some enzymatic activity X. It is noted that a cell can have the activity of any type of polypeptide reduced including, without limitation, enzymes, transcription factors, transporters, receptors, signal molecules, and the like. For example, a cell can contain an exogenous nucleic acid molecule that disrupts a regulatory and/or
15 coding sequence of a polypeptide having pyruvate decarboxylase activity or alcohol dehydrogenase activity. Disrupting pyruvate decarboxylase and/or alcohol dehydrogenase expression can lead to the accumulation of lactate as well as products produced from lactate such as 3-HP, 1,3-propanediol, acrylic acid, poly-acrylate, acrylate-esters, 3-HP-esters, and poly-3-HP. It is also noted that reduced polypeptide activities
20 can be the result of lower polypeptide concentration, lower specific activity of a polypeptide, or combinations thereof. Many different methods can be used to make a cell having reduced polypeptide activity. For example, a cell can be engineered to have a disrupted regulatory sequence or polypeptide-encoding sequence using common mutagenesis or knock-out technology. See, e.g., *Methods in Yeast Genetics* (1997
25 edition), Adams, Gottschling, Kaiser, and Sterns, Cold Spring Harbor Press (1998). Alternatively, antisense technology can be used to reduce the activity of a particular polypeptide. For example, a cell can be engineered to contain a cDNA that encodes an antisense molecule that prevents a polypeptide from being translated. The term
30 "antisense molecule" as used herein encompasses any nucleic acid molecule or nucleic acid analog (e.g., peptide nucleic acids) that contains a sequence that corresponds to the coding strand of an endogenous polypeptide. An antisense molecule also can have

flanking sequences (e.g., regulatory sequences). Thus, antisense molecules can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the molecule cleaves RNA. Further, gene silencing can be used to reduce the activity of a particular polypeptide.

A cell having reduced activity of a polypeptide can be identified using any method. For example, enzyme activity assays such as those described herein can be used to identify cells having a reduced enzyme activity.

A polypeptide having (1) the amino acid sequence set forth in SEQ ID NO:39 (the OS17 polypeptide) or (2) an amino acid sequence sharing at least about 60 percent sequence identity with the amino acid sequence set forth in SEQ ID NO:39 can have three functional domains: a domain having CoA-synthetase activity, a domain having 3-HP-CoA dehydratase activity, and a domain having CoA-reductase activity. Such polypeptides can be selectively modified by mutating and/or deleting domains such that one or two of the enzymatic activities are reduced. Reducing the dehydratase activity of the OS17 polypeptide can cause acrylyl-CoA to be created from propionyl-CoA. The acrylyl-CoA then can be contacted with a polypeptide having CoA hydrolase activity to produce acrylate from propionate (Figure 43). Similarly, acrylyl-CoA can be created from 3-HP by using, for example, an OS17 polypeptide having reduced reductase activity.

D. Production of Organic Acids and Related Products via In Vitro Techniques

In addition, purified polypeptides having enzymatic activity can be used alone or in combination with cells to produce 3-HP or other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP. For example, a preparation containing a substantially pure polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be used to catalyze the formation of 3-HP-CoA, a precursor to 3-HP. Further, cell-free extracts containing a polypeptide having enzymatic activity can be used alone or in combination with purified polypeptides and/or cells to produce 3-HP. For example, a cell-free extract containing a

polypeptide having CoA transferase activity can be used to form lactyl-CoA, while a microorganism containing polypeptides have the enzymatic activities necessary to catalyze the reactions needed to form 3-HP from lactyl-CoA can be used to produce 3-HP. Any method can be used to produce a cell-free extract. For example, osmotic shock, sonication, and/or a repeated freeze-thaw cycle followed by filtration and/or centrifugation can be used to produce a cell-free extract from intact cells.

It is noted that a cell, purified polypeptide, and/or cell-free extract can be used to produce 3-HP that is, in turn, treated chemically to produce another compound. For example, a microorganism can be used to produce 3-HP, while a chemical process is used to modify 3-HP into a derivative such as polymerized 3-HP or an ester of 3-HP. Likewise, a chemical process can be used to produce a particular compound that is, in turn, converted into 3-HP or other organic compound (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP) using a cell, substantially pure polypeptide, and/or cell-free extract described herein. For example, a chemical process can be used to produce acrylyl-CoA, while a microorganism can be used convert acrylyl-CoA into 3-HP.

E. Fermentation of Cells to Produce Organic Acids

Typically, 3-HP is produced by providing a production cell, such as a microorganism, and culturing the microorganism with culture medium such that 3-HP is produced. In general, the culture media and/or culture conditions can be such that the microorganisms grow to an adequate density and produce 3-HP efficiently. For large-scale production processes, any method can be used such as those described elsewhere (Manual of Industrial Microbiology and Biotechnology, 2nd Edition, Editors: A. L. Demain and J. E. Davies, ASM Press; and Principles of Fermentation Technology, P. F. Stanbury and A. Whitaker, Pergamon). Briefly, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing appropriate culture medium with, for example, a glucose carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be

larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose.

Once transferred, the microorganisms can be incubated to allow for the production of 3-HP. Once produced, any method can be used to isolate the 3-HP. For example, common separation techniques can be used to remove the biomass from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the 3-HP from the microorganism-free broth. In addition, 3-HP can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated.

F. Products Created From the Disclosed Biosynthetic Routes

The organic compounds produced from any of the steps provided in Figures 1-5, 43-44, 54, and 55 can be chemically converted into other organic compounds. For example, 3-HP can be hydrogenated to form 1,3 propanediol, a valuable polyester monomer. Hydrogenating an organic acid such as 3-HP can be performed using any method such as those used to hydrogenate succinic acid and/or lactic acid. For example, 3-HP can be hydrogenated using a metal catalyst. In another example, 3-HP can be dehydrated to form acrylic acid. Any method can be used to perform a dehydration reaction. For example, 3-HP can be heated in the presence of a catalyst (e.g., a metal or mineral acid catalyst) to form acrylic acid. Propanediol also can be created using polypeptides having oxidoreductase activity (e.g., enzymes is the 1.1.1.- class of enzymes) *in vitro* or *in vivo*.

V. Overview of Methodology Used to Create Biosynthetic Pathways That Make 3-HP from PEP

The invention provides methods of making 3-HP and related products from PEP via the use of biosynthetic pathways. Illustrative examples include methods involving the

production of 3-HP via a lactate intermediate, a malonyl-CoA intermediate, and a β -alanine intermediate.

A. Biosynthetic Pathway for Making 3-HP through a Lactic Acid Intermediate

5 A biosynthetic pathway that allows for the production of 3-HP from PEP was constructed (Figure 1). This pathway involved using several polypeptides that were cloned and expressed as described herein. *M. elsdenii* cells (ATCC 17753) were used as a source of genomic DNA. Primers were used to identify and clone a nucleic acid
10 sequence encoding a polypeptide having CoA transferase activity (SEQ ID NO: 1). The polypeptide was subsequently tested for enzymatic activity and found to have CoA transferase activity.

Similarly, PCR primers were used to identify nucleic acid sequences from *M. elsdenii* genomic DNA that encoded an E1 activator, E2 α , and E2 β polypeptides (SEQ
15 ID NOs: 9, 17, and 25, respectively). These polypeptides were subsequently shown to have lactyl-CoA dehydratase activity.

Chloroflexus aurantiacus cells (ATCC 29365) were used as a source of genomic DNA. Initial cloning lead to the identification of nucleic acid sequences: OS17 (SEQ ID NO: 129) and OS19 (SEQ ID NO: 40). Subsequence assays revealed that OS17 encodes
20 a polypeptide having CoA synthase activity, dehydratase activity, and dehydrogenase activity (propionyl-CoA synthetase). Subsequence assays also revealed that OS19 encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (also referred to as acrylyl-CoA hydratase activity).

Several operons were constructed for use in *E. coli*. These operons allow for the
25 production of 3-HP in bacterial cells. Additional experiments allowed for the expression of these polypeptide in yeast, which can be used to produce 3-HP.

B. Biosynthetic Pathway for Making 3-HP through a Malonyl-CoA Intermediate

30 Another pathway leading to the production of 3-HP from PEP was constructed. This pathway used a polypeptide having acetyl CoA carboxylase activity that was isolated

from *E. coli* (Example 9), and a polypeptide having malonyl-CoA reductase activity that was isolated from *Chloroflexus aurantiacus* (Example 10). The combination of these two polypeptides allows for the production of 3-HP from acetyl-CoA (Figure 44).

Nucleic acid encoding a polypeptide having malonyl-CoA reductase activity (SEQ ID NO:140) was cloned, sequenced, and expressed. The polypeptide having malonyl-CoA reductase activity was then used to make 3-HP.

C. Biosynthetic Pathways For Making 3-HP through a β -alanine Intermediate

In general, prokaryotes and eukaryotes metabolize glucose via the Embden-Meyerhof-Parnas pathway to PEP, a central metabolite in carbon metabolism. The PEP generated from glucose is either carboxylated to oxaloacetate or is converted to pyruvate. Carboxylation of PEP to oxaloacetate can be catalyzed by a polypeptide having PEP carboxylase activity, a polypeptide having PEP carboxykinase activity, or a polypeptide having PEP transcarboxylase activity. Pyruvate that is generated from PEP by a polypeptide having pyruvate kinase activity can also be converted to oxaloacetate by a polypeptide having pyruvate carboxylase activity.

Oxaloacetate generated either from PEP or pyruvate can act as a precursor for production of aspartic acid. This conversion can be carried out by a polypeptide having aspartate aminotransferase activity, which transfers an amino group from glutamate to oxaloacetate. Glutamate consumed in this reaction can be regenerated by the action of a polypeptide having glutamate dehydrogenase activity or by the action of a polypeptide having 4, 4-aminobutyrate aminotransferase activity. The decarboxylation of aspartate to β -alanine is catalyzed by a polypeptide having aspartate decarboxylase activity. β -alanine produced through this biochemistry can be converted to 3-HP via two possible pathways. These pathways are provided in Figures 54 and 55.

The steps involved in the production of β -alanine can be the same for both pathways. These steps can be accomplished by endogenous polypeptides in the host cells which convert PEP to β -alanine, or these steps can be accomplished with recombinant DNA technology using known polypeptides such as polypeptides having PEP-

carboxykinase activity (4.1.1.32), aspartate aminotransferase activity (2.6.1.1), and aspartate alpha-decarboxylase activity (4.1.1.11).

As depicted in Figure 54, a polypeptide having CoA transferase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:2) can be used to convert β -alanine to β -alanyl-CoA. β -alanyl-CoA can be converted to acrylyl-CoA via a polypeptide having β -alanyl-CoA ammonia lyase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:160). Acrylyl-CoA can be converted to 3-HP-CoA using a polypeptide having 3-HP-CoA dehydratase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:40). 3-HP-CoA can be converted into 3-HP via a polypeptide having CoA transferase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:2).

As depicted in Figure 55, a polypeptide having 4,4-aminobutyrate aminotransferase activity (2.6.1.19) can be used to convert β -alanine into malonate semialdehyde. The malonate semialdehyde can be converted into 3-HP using either a polypeptide having 3-hydroxypropionate dehydrogenase activity (1.1.1.59) or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

EXAMPLES

Example 1 – Cloning nucleic acid molecules that

encode a polypeptide having CoA transferase activity

Genomic DNA was isolated from *Megasphaera elsdenii* cells (ATCC 17753) grown in 1053 Reinforced Clostridium media under anaerobic conditions at 37°C in roll tubes for 12-14 hours. Once grown, the cells were pelleted, washed with 5 mL of a 10 mM Tris solution, and repelleted. The pellet was resuspended in 1 mL of Gentra Cell Suspension Solution to which 14.2 mg of lysozyme and 4 μ L of 20 mg/mL proteinase K solution was added. The cell suspension was incubated at 37°C for 30 minutes. The genomic DNA was then isolated using a Gentra Genomic DNA Isolation Kit following the provided protocol. The precipitated genomic DNA was spooled and air-dried for 10 minutes. The genomic DNA was suspended in 500 μ L of a 10 mM Tris solution and stored at 4°C.

Two degenerate forward (CoAF1 and CoAF2) and three degenerate reverse (CoAR1, CoAR2, and CoAR3) PCR primers were designed based on conserved acetoacetyl CoA transferase and propionate CoA transferase sequences (CoAF1 5'-GAAWSCGGYSCNATYGGYGG-3', SEQ ID NO: 49; CoAF2 5'-TTYTGYG-
5 GYRSBTTYACBGCWGG-3', SEQ ID NO: 50; CoAR1 5'-CCWGCVGTRAAV-SYRCCRCARAA-3', SEQ ID NO: 51; CoAR2 5'-AARACDSMRCGTTCVGTRA-TRTA-3', SEQ ID NO: 52; and CoAR3 5'-TCRAYRCCSGGWGCRAYTTC-3', SEQ ID NO: 53). The primers were used in all logical combinations in PCR using Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and 1 ng of genomic DNA
10 per μ L reaction mix. PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 59°C, 4 cycles at 57°C, 4 cycles at 55°C, and 18 cycles at 52°C. Each cycle used an initial 30-second denaturing step at 94°C and a 3 minute extension at 72°C. The program had an initial denaturing step for 2 minutes at 94°C and a final extension step of 4 minutes at 72°C. Time allowed for annealing was 45 seconds.
15 The amounts of PCR primer used in the reactions were increased 2-8 fold above typical PCR amounts depending on the amount of degeneracy in the 3' end of the primer. In addition, separate PCR reactions containing each individual primer were made to identify PCR products resulting from single degenerate primers. Each PCR product (25 μ L) was separated by electrophoresis using a 1% TAE (Tris-acetate-EDTA) agarose gel.
20 The CoAF1-CoAR2, CoAF1-CoAR3, CoAF2-CoAR2, and CoAF2-CoAR3 combinations produced a band of 423, 474, 177, and 228 bp, respectively. These bands matched the sizes based on other CoA transferase sequences. No band was visible from the individual primer control reactions. The CoAF1-CoAR3 fragment (474 bp) was isolated and purified using a Qiagen Gel Extraction Kit (Qiagen Inc., Valencia, CA).
25 Four μ L of the purified band was ligated into pCRII vector and transformed into TOP10 *E. coli* cells by heat-shock using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 μ g/mL of ampicillin (Amp) and 50 μ g/mL of 5-Bromo-4-Chloro-3-Indolyl-*B*-D-Galactopyranoside (X-gal). Single, white colonies were plated onto fresh media and screened in a PCR reaction using the
30 CoAF1 and CoAR3 primers to confirm the presence of the insert.

Plasmid DNA obtained using a QiaPrep Spin Miniprep Kit (Qiagen, Inc) was quantified and used for DNA sequencing with M13R and M13F primers. Sequence analysis revealed that the CoAF1-CoAR3 fragment shared sequence similarity with acetoacetyl CoA transferase sequences.

5 Genome walking was performed to obtain the complete coding sequence. The following primers for genome walking in both upstream and downstream directions were designed using the portion of the 474 bp CoAF1-CoAR3 fragment sequence that was internal to the degenerate primers (COAGSP1F 5'-GAATGTTTACTTCTGCGG-CACCTTCAC-3', SEQ ID NO:54; COAGSP2F 5'-GACCAGATCACTTTCAACG-GTTCCTATG-3', SEQ ID NO:55; COAGSP1R 5'-GCATAGGAACCGTTGAAA-GTGATCTGG-3', SEQ ID NO:56; and COAGSP2R 5'-GTTAGTACCGAACTTG-CTGACGTTGATG-3', SEQ ID NO:57). The COAGSP1F and COAGSP2F primers face downstream, while the COAGSP1R and COAGSP2R primers face upstream. In addition, the COAGSP2F and COAGSP2R primers are nested inside the COAGSP1F and
10 COAGSP1R primers. Genome walking was performed using the Universal Genome Walking kit (ClonTech Laboratories, Inc., Palo Alto, CA) with the exception that additional libraries were generated with enzymes *Nru* I, *Sca* I, and *Hinc* II. First round PCR was conducted in a Perkin Elmer 2400 Thermocycler with 7 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 65°C
15 with a final extension at 65°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. The first and second round product (20 µL) was separated by electrophoresis on a 1% TAE agarose gel. Amplification products were obtained with the *Stu* I library for the reverse direction. The second round
20 product of 1.5 Kb from this library was gel purified, cloned, and sequenced. Sequence analysis revealed that the sequence derived from genome walking overlapped with the CoAF1-CoAR3 fragment and shared sequence similarity with other sequences such as acetoacetyl CoA transferase sequences (Figures 8-9).

25 Nucleic acid encoding the CoA transferase (propionyl-CoA transferase or *pct*) from *Megasphaera elsdenii* was PCR amplified from chromosomal DNA using following PCR program: 25 cycles of 95°C for 30 seconds to denature, 50°C for 30 seconds to

anneal, and 72°C for 3 minutes for extension (plus 2 seconds per cycle). The primers used were designated PCT-1.114 (5'-ATGAGAAAAGTAGAAATCATTAC-3'; SEQ ID NO:58) and PCT-2.2045 (5'-GGCGGAAGTTGACGATAATG-3'; SEQ ID NO:59). The resulting PCR product (about 2 kb as judged by agarose gel electrophoresis) was purified using a Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified product was ligated to pETBlue-1 using the Perfectly Blunt cloning Kit (Novagen, Madison, WI). The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen, Madison, WI) that were spread on LB agar plates supplemented with 50 µg/mL carbenicillin, 40 µg/mL IPTG, and 40 µg/mL X-Gal. White colonies were isolated and screened for the presence of inserts by restriction mapping. Isolates with the correct restriction pattern were sequenced from each end using the primers pETBlueUP and pETBlueDOWN (Novagen) to confirm the sequence at the ligation points.

The plasmid was transformed into Tuner (DE3) pLacI chemically competent cells (Novagen, Madison, WI), and expression from the construct tested. Briefly, a culture was grown overnight to saturation and diluted 1:20 the following morning in fresh LB medium with the appropriate antibiotics. The culture was grown at 37°C with aeration to an OD₆₀₀ of about 0.6. The culture was induced with IPTG at a final concentration of 100 µM. The culture was incubated for an additional two hours at 37°C with aeration. Aliquots were taken pre-induction and 2 hours post-induction for SDS-PAGE analysis. A band of the expected molecular weight (55,653 Daltons predicted from the sequence) was observed after IPTG treatment. This band was not observed in cells containing a plasmid lacking the nucleic acid encoding the transferase.

Cell free extracts were prepared to assess enzymatic activity. Briefly, the cells were harvested by centrifugation and disrupted by sonication. The sonicated cell suspension was centrifuged to remove cell debris, and the supernatant was used in the assays.

Transferase activity was measured in the following assay. The assay mixture used contained 100 mM potassium phosphate buffer (pH 7.0), 200 mM sodium acetate, 1 mM dithiobisnitrobenzoate (DTNB), 500 µM oxaloacetate, 25 µM CoA-ester substrate, and 3 µg/mL citrate synthase. If present, the CoA transferase transfers the CoA from the CoA ester to acetate to form acetyl-CoA. The added citrate synthase condenses oxaloacetate

and acetyl-CoA to form citrate and free CoASH. The free CoASH complexes with DTNB, and the formation of this complex can be measured by a change in the optical density at 412 nm. The activity of the CoA transferase was measured using the following substrates: lactyl-CoA, propionyl-CoA, acrylyl-CoA, and 3-hydroxypropionyl-CoA. The units/mg of protein was calculated using the following formula:

$$(\Delta E/\text{min} * V_f * \text{dilution factor}) / (V_s * 14.2) = \text{units/mL}$$

where $\Delta E/\text{min}$ is the change in absorbance per minute at 412 nm, V_f is the final volume of the reaction, and V_s is the volume of sample added. The total protein concentration of the cell free extract was about 1 mg/mL so the units/mL equals units/mg.

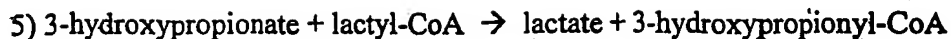
Cell free extracts from cells containing nucleic acid encoding the CoA transferase exhibited CoA transferase activity (Table 2). The observed CoA transferase activity was detected for the lactyl-CoA, propionyl-CoA, acrylyl-CoA, and 3-hydroxypropionyl-CoA substrates (Table 2). The highest CoA transferase activity was detected for lactyl-CoA and propionyl-CoA.

Table 2

Substrate	Units/mg
Lactyl-CoA	211
Propionyl-CoA	144
Acrylyl-CoA	118
3-Hydroxypropionyl-CoA	110

The following assay was performed to test whether the CoA transferase activity can use the same CoA substrate donors as recipients. Specifically, CoA transferase activity was assessed using a Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Voyager RP workstation (PerSeptive Biosystems). The following five reactions were analyzed:

- 1) acetate + lactyl-CoA \rightarrow lactate + acetyl-CoA
- 2) acetate + propionyl-CoA \rightarrow propionate + acetyl-CoA
- 3) lactate + acetyl-CoA \rightarrow acetate + lactyl-CoA
- 4) lactate + acrylyl-CoA \rightarrow acrylate + lactyl-CoA



MALDI-TOF MS was used to measure simultaneously the appearance of the product CoA ester and the disappearance of the donor CoA ester. The assay buffer contained 50 mM potassium phosphate (pH 7.0), 1 mM CoA ester, and 100 mM respective acid salt. Protein from a cell free extract prepared as described above was added to a final concentration of 0.005 mg/mL. A control reaction was prepared from a cell free extract prepared from cells lacking the construct containing the CoA transferase-encoding nucleic acid. For each reaction, the cell free extract was added last to start the reaction. Reactions were allowed to proceed at room temperature and were stopped by adding 1 volume 10% trifluoroacetic acid (TFA). The reaction mixtures were purified prior to MALDI-TOF MS analysis using Sep Pak Vac C₁₈ 50 mg columns (Waters, Inc.). The columns were conditioned with 1 mL methanol and equilibrated with two washes of 1 mL 0.1% TFA. Each sample was applied to the column, and the flow through was discarded. The column was washed twice with 1 mL 0.1% TFA. The sample was eluted in 200 μ L 40% acetonitrile, 0.1% TFA. The acetonitrile was removed by centrifugation *in vacuo*. Samples were prepared for MALDI-TOF MS analysis by mixing 1:1 with 110 mM sinapinic acid in 0.1% TFA, 67% acetonitrile. The samples were allowed to air dry.

In reaction #1, the control sample exhibited a main peak at a molecular weight corresponding to lactyl-CoA (MW 841). There was a minor peak at the molecular weight corresponding to acetyl-CoA (MW 811). This minor peak was determined to be the left-over acetyl-CoA from the synthesis of lactyl-CoA. The reaction #1 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited complete conversion of lactyl-CoA to acetyl-CoA. No peak was observed for lactyl-CoA.

This result indicates that the CoA transferase activity can transfer CoA from lactyl-CoA to acetate to form acetyl-CoA.

In reaction #2, the control sample exhibited a dominant peak at a molecular weight corresponding to propionyl-CoA (MW 825). The reaction #2 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a dominant peak at a molecular weight corresponding to acetyl-CoA (MW 811).

No peak was observed for propionyl-CoA. This result indicates that the CoA transferase activity can transfer CoA from propionyl-CoA to acetate to form acetyl-CoA.

5 In reaction #3, the control sample exhibited a dominant peak at a molecular weight corresponding to acetyl-CoA (MW 811). The reaction #3 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a peak corresponding to lactyl-CoA (MW 841). The peak corresponding to acetyl-CoA did not disappear. In fact, the ratio of the size of the two peaks was about 1:1. The observed appearance of the peak corresponding to lactyl-CoA demonstrates that the CoA transferase activity catalyzes reaction #3.

10 In reaction #4, the control sample exhibited a dominant peak at a molecular weight corresponding to acrylyl-CoA (MW 823). The reaction #4 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a dominant peak corresponding to lactyl-CoA (MW 841). This result demonstrates that the CoA transferase activity catalyzes reaction #4.

15 In reaction #5, deuterated lactyl-CoA was used to detect the transfer of CoA from lactate to 3-hydroxypropionate since lactic acid and 3-HP have the same molecular weight as do their respective CoA esters. Using deuterated lactyl-CoA allowed for the differentiation between lactyl-CoA and 3-hydroxypropionate using MALDI-TOF MS. The control sample exhibited a diffuse group of peaks at molecular weights ranging from
20 MW 841 to 845 due to the varying amounts of hydrogen atoms that were replaced with deuterium atoms. In addition, a significant peak was observed at a molecular weight corresponding to acetyl-CoA (MW 811). This peak was determined to be the left-over acetyl-CoA from the synthesis of lactyl-CoA. The reaction #5 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a
25 dominant peak at a molecular weight corresponding to 3-hydroxypropionyl-CoA (MW 841) as opposed to a group of peaks ranging from MW 841 to 845. This result demonstrates that the CoA transferase catalyzes reaction #5.

Example 2 – Cloning nucleic acid molecules that encode a
multiple polypeptide complex having lactyl-CoA dehydratase activity

The following methods were used to clone an E1 activator polypeptide. Briefly, four degenerate forward and five degenerate reverse PCR primers were designed based on conserved sequences of E1 activator protein homologs (E1F1 5'- GCWACBGGY-TAYGGYCG-3', SEQ ID NO:60; E1F2 5'-GTYRTYGAYRTYGGYGGYCAGGA-3', SEQ ID NO:61; E1F3 5'-ATGAACGAYAARTGYGCWGCWGG-3', SEQ ID NO:62; E1F4 5'-TGYGCWGCWGGYACBGGYCGYTT-3', SEQ ID NO:63; E1R1 5'-TCCT-GRCCRCCRAYRTCRAIRAC-3', SEQ ID NO:64; E1R2 5'-CCWGCWGCRCAY-TTRTCGTTTCAT-3', SEQ ID NO:65; E1R3 5'-AARCGRCCVGTGCCWGCWG-CRCA-3', SEQ ID NO:66; E1R4 5'- GCTTCGSWTTTCRACRATGSW-3', SEQ ID NO:67; and E1R5 5'-GSWRATRACCTTCGCWTTTCWGCRAA-3', SEQ ID NO:68).

The primers were used in all logical combinations in PCR using Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and 1 ng of genomic DNA per μ L reaction mix. PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 60°C, 4 cycles at 58°C, 4 cycles at 56°C, and 18 cycles at 54°C. Each cycle used an initial 30-second denaturing step at 94°C and a 3 minute extension step at 72°C. The program had an initial denaturing step for 2 minutes at 94°C and a final extension step of 4 minutes at 72°C. Time allowed for annealing was 45 seconds. The amounts of PCR primer used in the reactions were increased 2-10 fold above typical PCR amounts depending on the amount of degeneracy in the 3' end of the primer. In addition, separate PCR reactions containing each individual primer were made to identify PCR product resulting from single degenerate primers. Each PCR product (25 μ L) was separated by electrophoresis using a 1% TAE (Tris-acetate-EDTA) agarose gel.

The E1F2-E1R4, E1F2-E1R5, E1F3-E1R4, E1F3-E1R5, and E1F4-E1R4R2 combinations produced a band of 195, 207, 144, 156, and 144 bp, respectively. These bands matched the expected size based on E1 activator sequences from other species. No band was visible with individual primer control reactions. The E1F2-E1R5 fragment (207 bp) was isolated and purified using Qiagen Gel Extraction procedure (Qiagen Inc., Valencia, CA). The purified band (4 μ L) was ligated into a *pCRII* vector that then was transformed into TOP10 *E. coli* cells by heat-shock using a TOPO cloning procedure

(Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 $\mu\text{g/mL}$ of ampicillin (Amp) and 50 $\mu\text{g/mL}$ of 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside (X-gal). Single, white colonies were plated onto fresh media and screened in a PCR reaction using the E1F2 and E1R5 primers to confirm the presence of the insert. Plasmid DNA was obtained from multiple colonies using a QiaPrep Spin Miniprep Kit (Qiagen, Inc). Once obtained, the plasmid DNA was quantified and used for DNA sequencing with M13R and M13F primers. Sequence analysis revealed a nucleic acid sequence encoding a polypeptide and revealed that the E1F2-E1R5 fragment shared sequence similarity with E1 activator sequences (Figures 12-13).

Genome walking was performed to obtain the complete coding sequence of E2 α and β subunits. Briefly, four primers for performing genome walking in both upstream and downstream directions were designed using the portion of the 207 bp E1F2-E1R5 fragment sequence that was internal to the E1F2 and E1R5 degenerate primers (E1GSP1F 5'-ACGTCATGTCGAAGGTACTGGAAATCC-3', SEQ ID NO:69; E1GSP2F 5'-GGGACTGGTACTTCAAATCGAAGCATC-3', SEQ ID NO:70; E1GSP1R 5'-TGACGGCAGCGGGATGCTTCGATTTGA-3', SEQ ID NO:71; and E1GSP2R 5'-TCAGACATGGGGATTTCAGTACCTTC-3', SEQ ID NO:72). The E1GSP1F and E1GSP2F primers face downstream, while the E1GSP1R and E1GSP2R primers face upstream. In addition, the E1GSP2F and E1GSP2R primers are nested inside the E1GSP1F and E1GSP1R primers.

Genome walking was performed using the Universal Genome Walking Kit (ClonTech Laboratories, Inc., Palo Alto, CA) with the exception that additional libraries were generated with enzymes *Nru* I, *Sca* I, and *Hinc* II. First round PCR was performed in a Perkin Elmer 2400 Thermocycler with 7 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. The first and second round product (20 μL) was separated by electrophoresis using 1% TAE agarose gel. Amplification products were obtained with the *Stu* I library for both forward and reverse directions. The second round product of about 1.5 kb for forward direction and 3 kb fragment for reverse direction from the *Stu* I

library were gel purified, cloned, and sequenced. Sequence analysis revealed that the sequence derived from genome walking overlapped with the E1F2-E1R5 fragment.

To obtain additional sequence, a second genome walk was performed using a first round primer (E1GSPF5 5'-CCGTGTTACTTGGGAAGGTATCGCTGTCTG-3', SEQ ID NO:73) and a second round primer (E1GSPF6 5'-GCCAATGAAGGAGGAAA-CCACTAATGAGTC-3', SEQ ID NO:74). The genome walk was performed using the *Nru*I, *Sca*I, and *Hinc*II libraries. In addition, ClonTech's Advantage-Genomic Polymerase was used for the PCR. First round PCR was performed in a Perkin Elmer 2400 Thermocycler with an initial denaturing step at 94°C for 2 minutes, 7 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. The first and second round product (20 µL) was separated by electrophoresis on a 1% agarose gel. An about 1.5 kb amplification product was obtained from second round PCR of the *Hinc*II library. This band was gel purified, cloned, and sequenced. Sequence analysis revealed that it overlapped with the previously obtained genome walk fragment. In addition, sequence analysis revealed a nucleic acid sequence encoding an E2 α subunit that shares sequence similarities with other sequences (Figures 16-17). Further, sequence analysis revealed a nucleic acid sequence encoding an E2 β subunit that shares sequence similarities with other sequences (Figures 20-21).

Additional PCR and sequence analysis revealed the order of polypeptide encoding sequences within the region containing the lactyl-CoA dehydratase-encoding sequences. Specifically, the E1GSP1F and COAGSP1R primer pair and the COAGSP1F and E1GSP1R primer pair were used to amplify fragments that encode both the CoA transferase and E1 activator polypeptides. Briefly, *M. elsdenii* genome DNA (1 ng) was used as a template. The PCR was conducted in Perkin Elmer 2400 Thermocycler using Long Template Polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The PCR program used was as follows: 94°C for 2 minutes; 29 cycles of 94°C for 30 seconds, 61°C for 45 seconds, and 72°C for 6 minutes; and a final extension of 72°C for 10 minutes. Both PCR products (20 µL) were separated on a 1% agarose gel. An

amplification product (about 1.5 kb) was obtained using the COAGSP1F and E1GSP1R primer pair. This product was gel purified, cloned, and sequenced (Figure 22).

The organization of the *M. elsdenii* operon containing the lactyl-CoA dehydratase-encoding sequences was determined to containing the following polypeptide-encoding sequences in the following order: CoA transferase (Figure 6), ORFX (Figure 23), E1 activator protein of lactyl-CoA dehydratase (Figure 10), E2 α subunit of lactyl-CoA dehydratase (Figure 14), E2 β subunit of lactyl-CoA dehydratase (Figure 18), and truncated CoA dehydrogenase (Figure 25).

The lactyl-CoA dehydratase (lactyl-CoA dehydratase or *lcd*) from *M. elsdenii* was PCR amplified from chromosomal DNA using the following program: 94°C for 2 minutes; 7 cycles of 94°C for 30 seconds, 47°C for 45 seconds, and 72°C for 3 minutes; 25 cycles of 94°C for 30 seconds, 54°C for 45 seconds, and 72°C for 3 minutes; and 72°C for 7 minutes. One primer pair was used (OSNBE1F 5'-GGGAATTCATATG-AAAACTGTGTATACTCTC-3', SEQ ID NO:75 and OSNBE1R 5'-CGACGGAT-CCTTAGAGGATTTCCGAGAAAGC-3', SEQ ID NO:76). The amplified product (about 3.2 kb) was separated on 1% agarose gel, cut from the gel, and purified with a Qiagen Gel Extraction kit (Qiagen, Valencia, CA). The purified product was digested with *Nde* I and *Bam*HI restriction enzymes and ligated into pET11a vector (Novagen) digested with the same enzymes. The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen) that then were spread on LB agar plates supplemented with 50 μ g/mL carbenicillin. Isolated individual colonies were screened for the presence of inserts by restriction mapping. Isolates with the correct restriction pattern were sequenced from each end using Novagen primers (T7 promoter primer #69348-3 and T7 terminator primer #69337-3) to confirm the sequence at the ligation points.

A plasmid having the correct insert was transformed into Tuner (DE3) pLacI chemically competent cells (Novagen, Madison, WI). Expression from this construct was tested as follows. A culture was grown overnight to saturation and diluted 1:20 the following morning in fresh LB medium with the appropriate antibiotics. The culture was grown at 37°C with aeration to an OD₆₀₀ of about 0.6. The culture was induced with IPTG at a final concentration of 100 μ M. The culture was incubated for an additional two

hours at 37°C with aeration. Aliquots were taken pre-induction and 2 hours post-induction for SDS-PAGE analysis. Bands of the expected molecular weight (27,024 Daltons for the E1 subunit, 48,088 Daltons for the E2 α subunit, and 42,517 Daltons for the E2 β subunit—all predicted from the sequence) were observed. These bands were not observed in cells containing a plasmid lacking the nucleic acid encoding the three components of the lactyl-CoA dehydratase.

Cell free extracts were prepared by growing cells in a sealed serum bottle overnight at 37°C. Following overnight growth, the cultures were induced with 1 mM IPTG (added using anaerobic technique) and incubated an additional 2 hours at 37°C. The cells were harvested by centrifugation and disrupted by sonication under strict anaerobic conditions. The sonicated cell suspension was centrifuged to remove cell debris, and the supernatant was used in the assays. The buffer used for cell resuspension/sonication was 50 mM Tris-HCl (pH 7.5), 200 μ M ATP, 7 mM Mg(SO₄), 4 mM DTT, 1 mM dithionite, and 100 μ M NADH.

Dehydratase activity was detected with MALDI-TOF MS. The assay was conducted in the same buffer as above with 1 mM lactyl-CoA or 1 mM acrylyl-CoA added and about 5 mg/mL cell free extract. Prior to MALDI-TOF MS analysis, samples were purified using Sep Pak Vac C₁₈ columns (Waters, Inc.) as described in Example 1. The following two reactions were analyzed:

- 1) acrylyl-CoA \rightarrow lactyl-CoA
- 2) lactyl-CoA \rightarrow acrylyl-CoA

In reaction #1, the control sample exhibited a peak at a molecular weight corresponding to acrylyl-CoA (MW 823). The reaction #1 sample containing the cell extract from cells transfected with the dehydratase-encoding plasmid exhibited a major peak at a molecular weight corresponding to lactyl-CoA (MW 841). This result indicates that the dehydratase activity can convert acrylyl-CoA into lactyl-CoA.

To detect dehydratase activity on lactyl-CoA, reaction #2 was carried out in 80% D₂O. The control sample exhibited a peak at a molecular weight corresponding to lactyl-CoA (MW 841). The reaction #2 sample containing the cell extract from cells transfected with the dehydratase-encoding plasmid revealed a lactyl-CoA peak shifted to a deuterated

form. This result indicates that the dehydratase-enzyme is active on lactyl-CoA. In addition, the results from both reactions indicate that the dehydratase enzyme can catalyze the lactyl-CoA \leftrightarrow acrylyl-CoA reaction in both directions.

5 Example 3 – Cloning nucleic acid molecules that encode
 a polypeptide having 3-hydroxypropionyl CoA dehydratase activity

Genomic DNA was isolated from *Chloroflexus aurantiacus* cells (ATCC 29365). Briefly, *C. aurantiacus* cells in 920 Chloroflexus medium were grown in 50 mL cultures (Falcon 2070 polypropylene tubes) using an Innova 4230 Incubator, Shaker (New Brunswick Scientific; Edison, NJ) at 50°C with interior lights. Once grown, the cells
 10 were pelleted, washed with 5 mL of a 10 mM Tris solution, and re-pelleted. Genomic DNA was isolated from the pelleted cells using a Gentra Genomic "Puregene" DNA isolation kit (Gentra Systems; Minneapolis, MN). Briefly, the pelleted cells were resuspended in 1 mL Gentra Cell Suspension Solution to which 14.2 mg of lysozyme and
 15 4 μ L of 20 mg/mL proteinase K solution was added. The cell suspension was incubated at 37°C for 30 minutes. The precipitated genomic DNA was recovered by centrifugation at 3500 x g for 25 minutes and air-dried for 10 minutes. The genomic DNA was suspended in 300 μ L of a 10 mM Tris solution and stored at 4°C.

 The genomic DNA was used as a template in PCR amplification reactions with
 20 primers designed based on conserved domains of crotonase homologs and a *Chloroflexus aurantiacus* codon usage table. Briefly, two degenerate forward (CRF1 and CRF2) and three degenerate reverse (CRR1, CRR2, and CRR3) PCR primers were designed (CRF1 5'-AA YCGBCCVAARGCNCTSAAYGC-3', SEQ ID NO:77; CRF2: 5'-
 TTYGTBGCNGGYGCNGAYAT-3', SEQ ID NO:78; CRR1 5'-ATRTCNG-
 25 CRCCNGCVACRAA-3', SEQ ID NO:79; CRR2 5'-CCRCCRCCSAGNG-
 CRWARCCRTT-3', SEQ ID NO:80; and CRR3 5'-SSWNGCRATVCGRATRTCAC-
 3', SEQ ID NO:81).

 These primers were used in all logical combinations in PCR using Taq polymerase (Roche Molecular Biochemicals; Indianapolis, IN) and 1 ng of the genomic DNA per μ L
 30 reaction mix. The PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 61°C, 4 cycles at 59°C, 4 cycles at 57°C, 4 cycles at 55°C,

and 16 cycles at 52°C. Each cycle used an initial 30-second denaturing step at 94°C and a 3-minute extension step at 72°C. The program also had an initial denaturing step for 2 minutes at 94°C and a final extension step of 4 minutes at 72°C. The time allowed for annealing was 45 seconds. The amounts of PCR primer used in the reaction were
5 increased 4-12 fold above typical PCR amounts depending on the amount of degeneracy in the 3' end of the primer. In addition, separate PCR reactions containing each individual primer were performed to identify amplification products resulting from single degenerate primers. Each PCR product (25 µL) was separated by gel electrophoresis using a 1% TAE (Tris-acetate-EDTA) agarose gel.

10 The CRF1-CRR1 and CRF2-CRR2 combinations produced a unique band of about 120 and about 150 bp, respectively. These bands matched the expected size based on crotonase genes from other species. No 120 bp or 150 bp band was observed from individual primer control reactions. Both fragments (i.e., the 120 bp and 150 bp bands) were isolated and purified using the Qiagen Gel Extraction kit (Qiagen Inc., Valencia,
15 CA). Each purified fragment (4 µL) was ligated into *pCRII* vector that then was transformed into TOP10 *E. coli* cells by a heat-shock method using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 µg/mL of ampicillin (Amp) and 50 µg/mL of 5-Bromo-4-Chloro-3-Indolyl-*B*-D-Galactopyranoside (X-gal). Single, white colonies were plated onto fresh
20 media and screened in a PCR reaction using the CRF1 and CRR1 primers and the CRF2 and CRR2 primers to confirm the presence of the desired insert. Plasmid DNA was obtained from multiple colonies with the desired insert using a QiaPrep Spin Miniprep Kit (Qiagen, Inc.). Once obtained, the DNA was quantified and used for DNA sequencing with M13R and M13F primers. Sequence analysis revealed the presence of
25 two different clones from the PCR product of about 150 bp. Each shared sequence similarity with crotonase and hydratase sequences. The two clones were designated OS17 (157 bp PCR product) and OS19 (151 bp PCR product).

Genome walking was performed to obtain the complete coding sequence of OS17. Briefly, primers for conducting genome walking in both upstream and downstream
30 directions were designed using the portion of the 157 bp CRF2-CRR2 fragment sequence that was internal to the CRF2 and CRR2 degenerate primers (OS17F1 5'-CGCTG-

ATATTCGCCAGTTGCTCGAAG-3', SEQ ID NO:82; OS17F2 5'-CCCATCTTG-
 CTTTCCGCAAGATTGAGC-3', SEQ ID NO:83; OS17F3 5'-CAATGGCCCTGCCGA-
 ATAACGCCCATCT-3', SEQ ID NO:84; OS17R1 5'-CTTCGAGCAACTGGCGAA-
 TATCAGCG-3', SEQ ID NO:85; OS17R2 5'-GCTCAATCTTGCGGAAAGCAAG-
 5 ATGGG-3', SEQ ID NO:86; and OS17R3 5'-AGATGGGCGTTATTCGGCAGGGCC-
 ATTG-3', SEQ ID NO:87). The OS17F1, OS17F3, and OS17F2 primers face
 downstream, while the OS17R2, OS17R3, and OS17R1 primers face upstream.

Genome walking was conducted using the Universal Genome Walking kit
 (ClonTech Laboratories, Inc., Palo Alto, CA) with the exception that additional libraries
 10 were generated with enzymes *Nru* I, *Fsp* I, and *Hinc* II. The first round PCR was
 conducted in a Perkin Elmer 2400 Thermocycler with 7 cycles of 2 seconds at 94°C and 3
 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 66°C with a final
 extension at 66°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C
 and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 66°C with a
 15 final extension at 66°C for 4 minutes. The first and second round amplification product
 (5 µL) was separated by gel electrophoresis on a 1% TAE agarose gel. After the second
 round PCR, an amplification product of about 0.4 kb was obtained with the *Fsp* I library
 using the OS17R1 primer in the reverse direction, and an amplification product of about
 0.6 kb was obtained with the *Hinc* II library using the OS17F2 primer in the forward
 20 direction. These PCR products were cloned and sequenced.

Sequence analysis revealed that the sequences derived from genome walking
 overlapped with the CRF2-CRR2 fragment and shared sequence similarity with crotonase
 and hydratase sequences.

A second genome walking was performed to obtain additional sequences. Six
 25 primers were designed for this second genome walk (OS17F4 5'-AAGCTGGG-
 TCTGATCGATGCCATTGCTACC-3', SEQ ID NO:88; OS17F5 5'-CTCGATTATCG-
 CCCATCCACGTATCGAG-3', SEQ ID NO:89; OS17F6 5'-TGGATGCAATCCG-
 CTATGGCATTATCCACG-3', SEQ ID NO:90; OS17R4 5'-TCATTCAGTGCG-
 TTCACCGGCGGATTTGTC-3', SEQ ID NO:91; OS17R5 5'-TCGATCCGGAAGT-
 30 AGCGATAGCGTTCGATG-3', SEQ ID NO:92; and OS17R6 5'-CTTGGCTGCAAT-
 CTCTTCGAGCACTTCAGG-3', SEQ ID NO:93). The OS17F4, OS17F5, and OS17F6

primers faced downstream, while the OS17R4, OS17R5, and OS17R6 primers faced upstream.

The second genome walk was performed using the same methods described for the first genome walk. After the second round of walking, an amplification product of about 2.3 kb was obtained with a *Hinc II* library using the OS17R5 primer in the reverse direction, and an amplification product of about 0.6 kb was obtained with a *Pvu II* library using the OS17F5 primer in the forward direction. The PCR products were cloned and sequenced. Sequence analysis revealed that the sequences derived from the second genome walking overlapped with the sequence obtained during the first genome walking. In addition, the sequence analysis revealed a sequence with 3572 bp.

A BLAST search revealed that the polypeptide encoded by this sequence shares sequence similarity with polypeptides having three different activities. Specifically, the beginning of the OS17 encoded-polypeptide shares sequence similarity with CoA-synthetases, the middle region of the OS17 encoded-polypeptide shares sequence similarity with enoyl-CoA hydratases, and the end region of the OS17 encoded-polypeptide shares sequence similarity with CoA-reductases.

A third genome walk was performed using four primers (OS17UP-6 5'-CATCAGAGGTAATCACCACTCGTGCA-3', SEQ ID NO:94; OS17UP-7 5'-AAGTAGTAGGCCACCTCGTCGCCATA-3', SEQ ID NO:95; OS17DN-1 5'-GCCAATCAGGCGCTGATCTATGTTCT-3', SEQ ID NO:96; and OS17DN-2 5'-CTGATCTATGTTCTGGCCTCGGAGGT-3', SEQ ID NO:97). The OS17UP-6 and OS17UP-7 primers face upstream, while the OS17DN-1 and OS17DN-2 primers face downstream. The third genome walk yielded an amplification product of about 1.2 kb with a *Nru I* library using the OS17UP-7 primer in the reverse direction. In addition, amplification products of about 4 kb and about 1.1 kb were obtained with a *Hinc II* and *Fsp I* library, respectively, using the OS17DN-2 primer in the forward direction. Sequence analysis revealed a nucleic acid sequence encoding a polypeptide (Figures 27-28). The complete OS17 gene had 5466 nucleotides and encoded a 1822 amino acid polypeptide. The calculated molecular weight of the OS17 polypeptide from the sequence was 201,346 (pI=5.71).

A BLAST search analysis revealed that the product of the OS17 nucleic acid has three different activities based on sequence similarity to (1) CoA-synthetases at the beginning of the OS17 sequence, (2) 3-HP dehydratases in the middle of the OS17 sequence, and (3) CoA-reductases at the end of the OS17 sequence. Thus, the OS17 clone appeared to encode a single enzyme capable of catalyzing three distinct reactions leading to the direct conversion of 3-hydroxypropionate to propionyl CoA: 3-HP → 3-HP-CoA → acrylyl-CoA → propionyl-CoA.

The OS17 gene from *C. aurantiacus* was PCR amplified from chromosomal DNA using the following conditions: 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds to denature, 54°C for 30 seconds to anneal, and 68°C for 6 minutes for extension; followed by 68°C for 10 minutes for final extension. Two primers were used (OS17F 5'-GGGAATTCCATATGATCGACACTGCG-3', SEQ ID NO:136; and OS17R 5'-CGAAGGATCCAACGATAATCGGCTCAGCAC-3', SEQ ID NO:137). The resulting PCR product (~5.6 Kb) was purified using Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified product was digested with NdeI and BamHI restriction enzymes, heated at 80°C for 20 minutes to inactivate the enzymes; purified using Qiagen PCR purification kit, and ligated into a pET11a vector (Novagen, Madison, WI) previously digested with NdeI and BamHI enzymes. The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen, Madison, WI) that were spread on LB agar plates supplemented with 50 µg/mL carbenicillin. Individual transformants were screened by PCR amplification of the OS17 DNA with the OS17F and OS17R primers and conditions as described above directly from colonies cells. Clones that yielded the 5.6 Kb product were used for plasmid purification with Qiagen QiaPrep Spin Miniprep Kit (Qiagen, Inc). Resulting plasmids were transformed into *E. coli* BL21(DE3) cells, and OS17 polypeptide expression induced. The apparent molecular weight of the OS17 polypeptide according to SDS gel electrophoresis was about 190,000 Da.

To assay OS17 polypeptide function, a 100 mL culture of BL21-DE3/pET11a-OS17 cells was started using 1 mL of overnight grown culture as an inoculum. The culture was grown to an OD of 0.5-0.6 and was induced with 100 µM IPTG. After two and a half hours of induction, the cells were harvested by spinning at 8000 rpm in the

floor centrifuge. The cells were washed with 10 mM Tris-HCl (pH 7.8) and passed twice through a French Press at a gauge pressure of 1000 psi. The cell debris was removed by centrifugation at 15,000 rpm. The activity of the OS17 polypeptide was measured spectrophotometrically, and the products formed during this enzymatic transformation were detected by LC/MS. The assay mix was as follows (*J. Bacteriol.*, 181:1088-1098 (1999)):

	Reagent	Volume	Final Conc.
	Tris-HCl (1000 mM, 7.8 pH)	10 μ L	50 mM
10	MgCl ₂ (100mM)	10 μ L	5 mM
	ATP (30 mM)	20 μ L	3 mM
	KCl (100 mM)	20 μ L	10 mM
	CoASH (5 mM)	20 μ L	0.5 mM
	NAD(P)H	20 μ L	0.5 mM
15	3-hydroxypropionate	2 μ L	1 mM
	Protein extract (7 mg/mL)	20 (40) μ L	140 μ g
	DI water	78 (58) μ L	
	Total	200 μ L	

20 The initial rate of reaction was measured by monitoring the disappearance of NAD(P)H at 340 nm. The activity of the OS17 polypeptide was measured using 3-HP as the substrate. The units/mL of total protein was calculated using the formula set forth in Example 1. The activity of the expressed OS17 polypeptide was calculated to be 0.061 U/mL of total protein. The reaction products were purified using a Sep Pak Vac column

25 (Waters). The column was conditioned with 1 mL methanol and washed two times with 0.5 mL 0.1% TFA. The sample was then applied to the column, and the column was washed two more times with 0.5 mL 0.1% TFA. The sample was eluted with 200 μ L of 40% acetonitrile, 0.1% TFA. The acetonitrile was removed from the sample by vacuum centrifugation. The reaction products were analyzed by LC/MS.

30 Analyses of thioesters namely propionyl CoA, acrylyl CoA, and 3 HP CoA from the above reaction were carried out using a Waters/Micromass ZQ LC/MS instrument

which had a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) placed in series between the chromatograph and the single quadrupole mass spectrometer. LC separations were made using a 4.6 x 150 mm YMC ods-AQ (3 μ m particles, 120 Å pores) reversed-phase chromatography column at room temperature.

- 5 CoA esters were eluted in Buffer A (25 mM ammonium acetate, 0.5% acetic acid) with a linear gradient of buffer B (acetonitrile, 0.5% acetic acid). A flow rate of 0.25 mL/minute was used, and photodiode array UV absorbance was monitored from 200 to 400 nm. All parameters of the electrospray MS system were optimized and selected based on generation of protonated molecular ions ($[M+H]^+$) of the analytes of interest and
- 10 production of characteristic fragment ions. The following instrumental parameters were used for ESI-MS detection of CoA and organic acid-CoA thioesters in the positive ion mode; Extractor: 1 V; RF lens: 0 V; Source temperature: 100°C; Desolvation temperature: 300°C; Desolvation gas: 500 L/hour; Cone gas: 40 L/hour; Low mass resolution: 13.0; High mass resolution: 14.5; Ion energy: 0.5; Multiplier: 650.
- 15 Uncertainties for mass charge ratios (m/z) and molecular masses are $\pm 0.01\%$.

The enzyme assay mix from strains expressing the OS17 polypeptide exhibited peaks for propionyl CoA, acrylyl CoA, and 3-HP CoA with the propionyl CoA peak being the dominant peak. These peaks were missing in the enzyme assay mix obtained from the control strain, which carried vector pET11a without an insert. These results

20 indicate that the OS17 polypeptide has CoA synthetase activity, CoA hydratase activity, and dehydrogenase activity.

Genome walking also was performed to obtain the complete coding sequence of OS19. Briefly, primers for conducting genome walking in both upstream and downstream directions were designed using the portion of the 151 bp CRF2-CRR2

25 fragment sequence that was internal to the CRF2 and CRR2 degenerate primers (OS19F1 5'-GGCTGATATCAAAGCGATGGCCAATGC-3', SEQ ID NO:98; OS19F2 5'-CCAC-GCCTATTGATATGCTCACCAGTG-3', SEQ ID NO:99; OS19F3 5'-GCAAACCGG-TGATTGCTGCCGTGAATGG-3', SEQ ID NO:100; OS19R1 5'-GCATTGGCCAT-CGCTTTGATATCAGCC-3', SEQ ID NO:101; OS19R2 5'-CACTGGTGAGCATATC-

30 AATAGGCGTGG-3', SEQ ID NO:102; and OS19R3 5'-CCATTACGGCAGCAA-

TCACCGGTTTGC-3', SEQ ID NO:103). The OS19F1, OS19F2, and OS19F3 primers face downstream, while the OS19R1, OS19R2, and OS19R3 primers face upstream.

An amplification product of about 0.25 kb was obtained with the *Fsp* I library using the OS19R1 primer, while an amplification product of about 0.65 kb was obtained with the *Pvu* II library using the OS19R1 primer. In addition, an amplification product of about 0.4 kb was obtained with the *Pvu* II library using the OS19F3 primer. The PCR products were cloned and sequenced. Sequence analysis revealed that the sequences derived from genome walking overlapped with the CRF2-CRR2 fragment and shared sequence similarity with crotonase and hydratase sequences. The obtained sequences accounted for most of the coding sequence including the start codon.

A second genome walk was performed to obtain additional sequence using two primers (OS19F7 5'-TCATCATCGCCAGTGAAAACGCGCAGTTTCG-3', SEQ ID NO:104 and OS19F8 5'-GGATCGCGCAAACCATTTGCCACCAAATCAC-3', SEQ ID NO:105). The OS19F7 and OS19F8 primers face downstream.

An amplification product (about 0.7 kb) obtained from the *Pvu* II library was cloned and sequenced. Sequence analysis revealed that the sequence derived from the second genome walk overlapped with the sequence obtained from the first genome walk and contained the stop codon. The full-length OS19 clone was found to share sequence similarity with other sequences such as crotonase and enoyl-CoA hydratase sequences (Figures 32-33).

The OS19 clone was found to encode a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity also referred to as acrylyl-CoA hydratase activity. The nucleic acid encoding the OS19 dehydratase from *C. aurantiacus* was PCR amplified from chromosomal DNA using the following conditions: 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds to denature, 56°C for 30 seconds to anneal, and 68°C for 1 minute for extension; and 68°C for 5 minutes for final extension. Two primers were used (OSACH3 5'-ATGAGTGAAGAGTCTCTGGTTCTCAGC-3', SEQ ID NO:106 and OSACH2 5'-AGATCGCAATCGCTCGTGTATGTC-3', SEQ ID NO:107).

The resulting PCR product (about 1.2 kb) was separated by agarose gel electrophoresis and purified using Qiagen PCR purification kit (Qiagen Inc.; Valencia, CA). The purified product was ligated into pETBlue-1 using the Perfectly Blunt cloning

Kit (Novagen; Madison, WI). The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen, Madison, WI) that then were spread on LB agar plates supplemented with 50 µg/mL carbenicillin, 40 µg/mL IPTG, and 40 µg/mL X-Gal. White colonies were isolated and screened for the presence of inserts by restriction mapping. Isolates with the correct restriction pattern were sequenced from each end using the primer pETBlueUP and pETBlueDOWN (Novagen) to confirm the sequence at the ligation points.

The plasmid containing the OS19 dehydratase-encoding sequence was transformed into Tuner (DE3) pLacI chemically competent cells (Novagen, Madison, WI), and expression from the construct tested. Briefly, a culture was grown overnight to saturation and diluted 1:20 the following morning in fresh LB medium with the appropriate antibiotics. The culture was grown at 37°C and 250 rpm to an OD₆₀₀ of about 0.6. At this point, the culture was induced with IPTG at a final concentration of 1 mM. ~~The culture was incubated for an additional two hours at 37°C and 250 rpm.~~ Aliquots were taken pre-induction and 2 hours post-induction for SDS-PAGE analysis. A band of the expected molecular weight (27,336 Daltons predicted from the sequence) was observed. This band was not observed in cells containing a plasmid lacking the nucleic acid encoding the hydratase.

~~Cell free extracts were prepared by growing cells as described above.~~ The cells were harvested by centrifugation and disrupted by sonication. The sonicated cell suspension was centrifuged to remove cell debris, and the supernatant was used in the assays. The ability of the 3-hydroxypropionyl-CoA dehydratase to perform the following three reactions was measured using MALDI-TOF MS:

- 1) acrylyl-CoA → 3-hydroxypropionyl-CoA
- 2) 3-hydroxypropionyl-CoA → acrylyl-CoA
- 3) crotonyl-CoA → 3-hydroxybutyryl-CoA

The assay mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM CoA ester, and about 1 µg cell free extract. Reactions were allowed to proceed at room temperature and were stopped by adding 1 volume 10% trifluoroacetic acid (TFA). The reaction mixtures were purified prior to MALDI-TOF MS analysis using Sep Pak Vac C₁₈ 50 mg columns

(Waters, Inc.). The columns were conditioned with 1 mL methanol and then equilibrated with two washes of 1 mL 0.1% TFA. The sample was applied to the column, and the flow through was discarded. The column was washed twice with 1 mL 0.1% TFA. The sample was eluted in 200 μ L 40% acetonitrile, 0.1% TFA. The acetonitrile was removed by centrifugation *in vacuo*. Samples were prepared for MALDI-TOF MS analysis by mixing 1:1 with 110 mM sinapinic acid in 0.1% TFA, 67% acetonitrile. The samples were allowed to air dry.

The conversion of acrylyl-CoA into 3-hydroxypropionyl-CoA catalyzed by the 3-hydroxypropionyl-CoA dehydratase was detected using the MALDI-TOF MS technique. In reaction #1, the control sample exhibited a dominant peak at a molecular weight corresponding to acrylyl-CoA (MW 823). The reaction #1 sample containing the cell extract from cells transfected with the 3-hydroxypropionyl-CoA dehydratase-encoding plasmid exhibited a dominant peak corresponding to 3-hydroxypropionyl-CoA (MW 841). This result demonstrates that the 3-hydroxypropionyl-CoA dehydratase activity catalyzes reaction #1.

To detect the conversion of 3-hydroxypropionyl-CoA into acrylyl-CoA, reaction #2 was carried out in 80% D₂O. The reaction #2 sample containing the cell extract from cells transfected with the 3-hydroxypropionyl-CoA dehydratase-encoding plasmid revealed incorporation of deuterium in the 3-hydroxypropionyl-CoA molecule. This result indicates that the 3-hydroxypropionyl-CoA dehydratase enzyme catalyzes reaction #2. In addition, the results from both #1 and #2 reactions indicate that the 3-hydroxypropionyl-CoA dehydratase enzyme can catalyze the 3-hydroxypropionyl-CoA \leftrightarrow acrylyl-CoA reaction in both directions. It is noted that for both the #1 and #2 reactions, a peak was observed at MW 811, due to leftover acetyl-CoA from the synthesis of 3-hydroxypropionyl-CoA from 3-hydroxypropionate and acetyl-CoA.

The assays assessing conversion of crotonyl-CoA into 3-hydroxybutyryl-CoA also were carried out in 80% D₂O. In reaction #3, the control sample exhibited a dominant peak at a molecular weight corresponding to crotonyl-CoA (MW 837). This result indicated that the crotonyl-CoA was not converted into other products. The reaction #3 sample containing the cell extract from cells transfected with the 3-hydroxypropionyl-CoA dehydratase-encoding plasmid exhibited a diffuse group of peaks corresponding to

deuterated 3-hydroxybutyryl-CoA (MW 855 to MW 857). This result demonstrates that the 3-hydroxypropionyl-CoA dehydratase activity catalyzes reaction #3.

A series of control reactions were performed to confirm the specificity of the 3-hydroxypropionyl-CoA dehydratase. Lactyl-CoA (1 mM) was added to the reaction mixture containing 100 mM Tris (pH 7.0) both in the presence and the absence of the 3-hydroxypropionyl-CoA dehydratase. In both cases, the dominant peak observed had a molecular weight corresponding to lactyl-CoA (MW 841). This result indicates that lactyl-CoA is not affected by the presence of 3-hydroxypropionyl-CoA dehydratase activity even in the presence of D₂O meaning that the 3-hydroxypropionyl-CoA dehydratase enzyme does not attach a hydroxyl group at the alpha carbon position. The presence of 3-hydroxypropionyl-CoA in an 80% D₂O reaction mixture resulted in a shift upon addition of the 3-hydroxypropionyl-CoA dehydratase activity. In the absence of 3-hydroxypropionyl-CoA dehydratase activity, a peak corresponding to 3-hydroxypropionyl-CoA was observed in addition to a peak of MW 811. The MW 811 peak was due to leftover acetyl-CoA from the synthesis of 3-hydroxypropionyl-CoA. In the presence of 3-hydroxypropionyl-CoA dehydratase activity, a peak corresponding to deuterated 3-hydroxypropionyl-CoA was observed (MW 842) due to exchange of a hydroxyl group during the conversion of 3-hydroxypropionyl-CoA to acrylyl-CoA and visa-versa. These control reactions demonstrate that the 3-hydroxypropionyl-CoA dehydratase enzyme is active on 3-hydroxypropionyl-CoA and not active on lactyl-CoA. In addition, these results demonstrate that the product of the acrylyl-CoA reaction is 3-hydroxypropionyl-CoA not lactyl-CoA.

Example 4 - Construction of operon #1

The following operon was constructed and can be used to produce 3-HP in *E. coli* (Figure 34). Briefly, the operon was cloned into a pET-11a expression vector under the control of a T7 promoter (Novagen, Madison, WI). The pET-11a expression vector is a 5677 bp plasmid that uses the ATG sequence of an *NdeI* restriction site as a start codon for inserted downstream sequences.

Nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were

used to amplify the CoA transferase-encoding sequence (OSNBpctF 5'-GGGAATTCC-ATATGAGAAAAGTAGAAATCATTACAGCTG-3', SEQ ID NO:108 and OSCTE-2 5'-GAGAGTATACACAGTTTTTCACCTCCTTTACAGCAGAGAT-3', SEQ ID NO:109), and two primers were used to amplify the lactyl-CoA dehydratase-encoding sequence (OSCTE-1 5'-ATCTCTGCTGTAAAGGAGGTGAAAAGTGTGTATACT-CTC-3', SEQ ID NO:110 and OSEBH-2 5'-ACGTTGATCTCCTTGTACATT-AGAGGATTTCCGAGAAAGC-3', SEQ ID NO:111). A nucleic acid molecule encoding a 3-hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSEBH-1 5'-GCTTTCTCGG-AAATCCTCTAATGTACAAGGAGATCAACGT-3', SEQ ID NO:112 and OSHBR 5'-CGACGGATCCTCAACGACCACTGAAGTTGG-3', SEQ ID NO:113).

PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The CoA transferase, lactyl-CoA dehydratase (E1, E2 α subunit, and E2 β subunit), and 3-hydroxypropionyl-CoA dehydratase PCR products were assembled using PCR. The OSCTE-1 and OSCTE-2 primers as well as the OSEBH-1 and OSEBH-2 primers were complementary to each other. Thus, the complementary DNA ends could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSHBR) were added to the assembly PCR mixture, which contained 100 ng of each PCR product (i.e., the PCR products from the CoA-transferase, lactyl-CoA dehydratase, and 3-hydroxypropionyl-CoA dehydratase reactions) as well as the rTth polymerase/Pfu Turbo polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 6 minutes; and a final extension at 68°C for 7 minutes. The assembled PCR

product was gel purified and digested with restriction enzymes (*NdeI* and *BamHI*). The sites for these restriction enzymes were introduced into the assembled PCR product using the OSNBpctF (*NdeI*) and OSHBR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactive the restriction enzymes and used directly for
5 ligation into pET-11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase
10 (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once heat shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and analyzed by
15 digestion with *NdeI* and *BamHI* restriction enzymes.

Example 5 - Construction of operon #2

The following operon was constructed and can be used to produce 3-HP in *E. coli* (Figure 35A and B). Nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR.
20 Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSCTE-2), and two primers were used to amplify the lactyl-CoA dehydratase-encoding sequence (OSCTE-1 and OSNBe1R 5'-CGACGGATCCTTAGAGGATTT-CCGAGAAAGC-3', SEQ ID NO:114). A nucleic acid molecule encoding a 3-
25 hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSXNhF 5'-GGTGTCT-AGAGACAGTCCTGTCGTTTATGTAGAAGGAG-3', SEQ ID NO:115 and OSXNhR 5'-GGGAATTCCATATGCGTAACTTCCTCCTGCTATCAACGACCACTGAA-GTTGG-3', SEQ ID NO:116).

30 PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and

Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The
5 obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The CoA transferase and lactyl-CoA dehydratase (E1, E2 α subunit, and E2 β subunit) PCR products were assembled using PCR. The OSCTE-1 and OSCTE-2 primers were complementary to each other. Thus, the 22 nucleotides at the end of the CoA
10 transferase sequence and the 22 nucleotides at the beginning of the lactyl-CoA dehydratase could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSNBelR) were added to the assembly PCR mixture, which contained 100 ng of the CoA transferase PCR product, 100 ng of lactyl-CoA dehydratase PCR product, and the
15 rTth polymerase/Pfu Turbo polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 5 minutes; and a final extension at 68°C for 6 minutes.

The assembled PCR product was gel purified and digested with restriction
20 enzymes (*NdeI* and *BamHI*). The sites for these restriction enzymes were introduced into the assembled PCR product using the OSNBpctF (*NdeI*) and OSNBelR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET-11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel
25 purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a
30 heat-shock method. Once heat shocked, the cells were plated on LB plates supplemented with 50 μ g/mL carbenicillin. The plasmid DNA was purified from individual colonies

using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and analyzed by digestion with *NdeI* and *BamHI* restriction enzymes. The digest revealed that the DNA fragment containing CoA transferase-encoding and lactyl-CoA dehydratase-encoding sequences was cloned into the pET-11a vector.

5 The plasmid carrying the CoA transferase-encoding and lactyl-CoA dehydratase-encoding sequences (pTD) was digested with *XbaI* and *NdeI* restriction enzymes, gel purified, and used for cloning the 3-hydroxypropionyl-CoA dehydratase-encoding product upstream of the CoA transferase-encoding sequence. Since this *XbaI* and *NdeI* digest eliminated a ribosome-binding site (RBS) from the pET-11a vector, a new
10 homologous RBS was cloned into the plasmid together with the 3-hydroxypropionyl-CoA dehydratase-encoding product. Briefly, the 3-hydroxypropionyl-CoA dehydratase-encoding PCR product was digested with *XbaI* and *NdeI* restriction enzymes, heated at 65°C for 30 minutes to inactivate the restriction enzymes, and ligated into pTD. The
ligation mixture was transformed into chemically competent NovaBlue cells (Novagen)
15 that then were plated on LB plates supplemented with 50 µg/mL carbenicillin.

Individual colonies were selected, and the plasmid DNA obtained using a Qiagen Spin Miniprep Kit. The obtained plasmids were digested with *XbaI* and *NdeI* restriction enzymes and analyzed by gel electrophoresis. pTD plasmids containing the inserted 3-hydroxypropionyl-CoA dehydratase-encoding PCR product were named pHTD. While
20 expression of the lactyl-CoA hydratase, CoA transferase, and 3-hydroxypropionyl-CoA dehydratase sequences from pHTD was directed by a single T7 promoter, each coding sequence had an individual RBS upstream of their start codon.

To ensure the correct assembly and cloning of the lactyl-CoA hydratase, CoA transferase, and 3-hydroxypropionyl-CoA dehydratase sequences into one operon, both
25 ends of the operon and all junctions between the coding sequences were sequenced. This DNA analysis revealed that the operon was assembled correctly.

The pHTD plasmid was transformed into BL21(DE3) cells to study the expression of the encoded sequences.

Example 6 - Construction of operons #3 and #4

Operon #3 (Figure 36A and B) and operon #4 (Figure 37A and B) each position the E1 activator at the end of the operon. Operon #3 contains a RBS between the 3-hydroxypropionyl-CoA dehydratase-encoding sequence and the E1 activator-encoding sequence. In operon #4, however, the stop codon of the 3-hydroxypropionyl-CoA dehydratase-encoding sequence is fused with the start codon of the E1 activator-encoding sequence as follows: TAGTG. The absence of the RBS in operon #4 can decrease the level of E1 activator expression.

To construct operon #3, nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSHTR 5'-ACGTTGATCTCCTTCTACATTATTTTTCAGT-CCCATG-3', SEQ ID NO:117), two primers were used to amplify the E2 α and β subunits of the lactyl-CoA dehydratase-encoding sequence (OSEIIXNF 5'-GGTGTCTAGAGTCAAAGGAGAGAAACAAAATCATGAGTG-3', SEQ ID NO:118 and OSEIIXNR 5'-GGGAATTCCATATGCGTAACTTCCTCCTGCTATTAGAGGATTTCCGAGAAAGC-3', SEQ ID NO:119), and two primers were used to amplify the E1 activator of the lactyl-CoA dehydratase-encoding sequence (OSHrEIF 5'-TCAGTG-GTCGTTGATCACGCTATAAAGAAAGGTGAAACTGTGTATACTCTC-3', SEQ ID NO:120 and OSEIBR 5'-CGACGGATCCCTTCCTTGGAGCTCATGCTTTC-3', SEQ ID NO:121). A nucleic acid molecule encoding a 3-hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSTHF 5'-CATGGGACTGAAAAATAATGTAGAAGGAGATCAACGT-3', SEQ ID NO:122 and OSElHR 5'-GAGAGTATACACAGTTTTCA-CCTTTCTTTATAGCGTGATCAACGACCACTGA-3', SEQ ID NO:123).

PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The

obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The 3-hydroxypropionyl-CoA dehydratase and E1 activator PCR products were assembled using PCR. The OSHrE1F and OSEIrHR primers were complementary to each other. Thus, the primers could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSTHF and OSE1BR) were added to the assembly PCR mixture, which contained 100 ng of the 3-hydroxypropionyl-CoA dehydratase PCR product, 100 ng of E1 activator PCR product, and the rTth polymerase/Pfu Turbo polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 1.5 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and used in a second assembly PCR with gel purified the CoA transferase PCR product. The OSTHF and OSHTR primers were complementary to each other. Thus, the complementary DNA ends could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSEIBR) were added to the second assembly PCR mixture, which contained 100 ng of the purified 3-hydroxypropionyl-CoA dehydratase/E1 PCR assembly, 100 ng of the purified CoA transferase PCR product, and the polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 3 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and digested with *NdeI* and *BamHI* restriction enzymes. The sites for these restriction enzymes were introduced into the assembled PCR products with the OSNBpctF (*NdeI*) and OSEIBR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactive the restriction enzymes and used directly for ligation into a pET11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the

assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once heat shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA). The resulting plasmids carrying the CoA transferase, 3-hydroxypropionyl-CoA dehydratase, and EI activator sequences (pThrEI) were digested with *XbaI* and *NdeI*, purified using gel electrophoresis and a Qiagen Gel Extraction kit, and used as a vector for cloning of the E2 α subunit/E2 β subunit PCR product.

The E2 α subunit/E2 β subunit PCR product was digested with the same enzymes and ligated into the pThrEI vector. The ligation reaction was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and digested with *XbaI* and *NdeI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the constructed operon #3 (pEIITHrEI) were transformed into BL21(DE3) cells to study the expression of the cloned sequences. Electrospray mass spectrometry assay confirmed that extracts from these cells have CoA transferase activity and 3-hydroxypropionyl-CoA dehydratase activity. Similar assays are used to confirm that extracts from these cells also have lactyl-CoA dehydratase activity.

To construct operon #4, nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSHTR), two primers were used to amplify the E2 α and β subunits of the lactyl-CoA dehydratase-encoding sequence (OSEIIXNF and OSEIIXNR), and two primers were used to amplify the E1 activator of the lactyl-CoA dehydratase-encoding sequence (OSHEIF 5'-CCAACTTCAGTGGTCGTTAGTGAAAAGTGTGTAT-ACTCTC-3', SEQ ID NO:124 and OSEIBR). A nucleic acid molecule encoding a 3-

hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSTHF and OSEIHR 5'-GAGAGTATACACAGTTTTCTACTAACGACCACTGAAGTTGG-3', SEQ ID NO:125).

5 PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C
10 for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The 3-hydroxypropionyl-CoA dehydratase and E1 activator PCR products were assembled using PCR. The OSHEIF and OSEIHR primers were complementary to each
15 other. Thus, the primers could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSTHF and OSE1BR) were added to the assembly PCR mixture, which contained 100 ng of the 3-hydroxypropionyl-CoA dehydratase PCR product, 100 ng of E1 activator PCR product, and the rTth polymerase/Pfu Turbo polymerase mix described above. The
20 following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 1.5 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and used in a second assembly PCR with gel purified the CoA transferase PCR product. The OSTHF and OSHTR primers
25 were complementary to each other. Thus, the complementary DNA ends could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSEIBR) were added to the second assembly PCR mixture, which contained 100 ng of the purified 3-hydroxypropionyl-CoA dehydratase/EI PCR assembly, 100 ng of the purified CoA
30 transferase PCR product, and the polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for

30 seconds, 54°C for 30 seconds, and 68°C for 3 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and digested with *NdeI* and *BamHI* restriction enzymes. The sites for these restriction enzymes were introduced into the assembled PCR products with the OSNBpctF (*NdeI*) and OSEIBR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). The resulting plasmids carrying the CoA transferase, 3-hydroxypropionyl-CoA dehydratase, and EI activator sequences (pTHE1) were digested with *XbaI* and *NdeI*, purified using gel electrophoresis and a Qiagen Gel Extraction kit, and used as a vector for cloning of the E2 α subunit/E2 β subunit PCR product.

The E2 α subunit/E2 β subunit PCR product was digested with the same enzymes and ligated into the pTHE1 vector. The ligation reaction was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and digested with *XbaI* and *NdeI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the constructed operon #4 (pEIITHEI) were transformed into BL21(DE3) cells to study the expression of the cloned sequences. Electrospray mass spectrometry assays confirmed that extracts from these cells have CoA transferase activity and 3-hydroxypropionyl-CoA dehydratase activity.

Similar assays are used to confirm that extracts from these cells also have lactyl-CoA dehydratase activity.

E. coli plasmid pEITHrEI carrying a synthetic 3-HP operon was digested with *Nru*I, *Xba*I and *Bam*HI restriction enzymes, *Xba*I-*Bam*HI DNA fragment was gel purified
5 with Quagen Gel Extraction Kit (Qiagen, Inc., Valencia CA) and used for further cloning into Bacillu vector pWH1520 (MoBiTec BmbH, Gottingen, Germany). Vector pWH1520 was digested with *Spe*I and *Bam*HI restriction enzymes and gel purified with Qiagen Gel Extraction Kit. The *Xba*I-*Bam*HI fragment carrying 3-HP operon was ligated into WH1520 vector at 16°C overnight using T4 ligase. The ligation mixture was
10 transformed into chemically competent TOP 10 cells and plated on LB plates supplemented with 50 µg/ml carbenicillin. One clone named *B. megaterium* (pBPO26) was used for assays of CoA-transferase and CoA-hydratase activities. The assays were performed as described above for *E. Coli*. The enzymatic activity was 5 U/mg and 13 U/mg respectively.

15

Example 7 - Construction of a two plasmid system

The following constructs were constructed and can be used to produce 3-HP in *E. coli* (Figure 38A and B). Nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR.
20 Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSHTR), two primers were used to amplify the E2 α and β subunits of the lactyl-CoA dehydratase-encoding sequence (OSEIIXNF and OSEIIXNR), and two primers were used to amplify the E1 activator of the lactyl-CoA dehydratase-encoding sequence (E1PROF 5'-GTCGCAGAATTCCCATCAATCGCAGCAATCCCAAC-3', SEQ ID
25 NO:126 and E1PROR 5'-TAACATGGTACCGACAGAAGCGGACCAGCA-AACGA-3', SEQ ID NO:127). A nucleic acid molecule encoding a 3-hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSTHF and OSHBR 5'-CGACGGATCCTCAACGACCA-CTGAAGTTGG-3', SEQ ID NO:128).

30 PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and

Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The
5 obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The CoA transferase PCR product and the 3-hydroxypropionyl-CoA dehydratase PCR product were assembled using PCR. The OSTHF and OSHTR primers were complementary to each other. Thus, the complementary DNA ends could anneal to each
10 other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSHBR) were added to the assembly PCR mixture, which contained 100 ng of the purified CoA transferase PCR product, 100 ng of the purified 3-hydroxypropionyl-CoA dehydratase PCR product, and the polymerase mix described above. The following PCR conditions were used to
15 assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2.5 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and digested with *NdeI* and *BamHI* restriction enzymes. The sites for these restriction enzymes were introduced into the assembled PCR products with the OSNBpctF (*NdeI*) and OSHBR (*BamHI*) primers. The
20 digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the
25 assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a
30 QiaPrep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA) and digested with *NdeI* and *BamHI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids

carrying the CoA transferase and 3-hydroxypropionyl-CoA dehydratase (pTH) were digested with *XbaI* and *NdeI*, purified using gel electrophoresis and a Qiagen Gel Extraction kit, and used as a vector for cloning of the E2 α subunit/E2 β subunit PCR product.

5 The E2 α subunit/E2 β subunit PCR product digested with the same enzymes was ligated into the pTH vector. The ligation reaction was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 μ g/mL carbenicillin. The plasmid DNA was purified
10 from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA) and digested with *XbaI* and *NdeI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the E2 α and β subunits of the lactyl-CoA dehydratase, the CoA transferase, and the 3-hydroxypropionyl-CoA dehydratase (pEITH) were transformed into BL21(DE3) cells to study the expression of the cloned sequences.

15 The gel purified E1 activator PCR product was digested with *EcoRI* and *KpnI* restriction enzymes, heated at 65°C for 30 minutes, and ligated into a vector (pPROLar.A) that was digested with *EcoRI* and *KpnI* restriction enzymes, gel purified using Qiagen Gel Extraction kit, and treated with shrimp alkaline phosphatase (Roche
20 Molecular Biochemicals; Indianapolis, IN). The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into DH10B electro-competent cells (Gibco Life Technologies; Gaithersburg, MD) using electroporation. Once electroporated, the cells were plated on LB plates supplemented with 25 μ g/mL kanamycin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen
25 Inc., Valencia, CA) and digested with *EcoRI* and *KpnI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the E1 activator (pPROEI) are transformed into BL21(DE3) cells to study the expression of the cloned sequence.

30 The pPROEI and pEITH plasmids are compatible plasmids that can be used in the same bacterial host cell. In addition, the expression from the pPROEI and pEITH plasmids can be induced at different levels using IPTG and arabinose, allowing for the fine-tuning of the expression of the cloned sequences.

Example 8 – Production of 3-HP

3-HP was produced using recombinant *E. coli* in a small-scale batch fermentation reaction. The construction of strain ALS848 (also designated as TA3476 (*J. Bacteriol.*, 143:1081-1085(1980))) that carried inducible T7 RNA polymerase was performed using

5 λ DE3 lysogenization kit (Novagen, Madison, WI) according to the manufacture's instructions. The constructed strain was designated ALS484(DE3). Strain ALS484(DE3) was transformed with pEIITHrEI plasmid using standard electroporation techniques. The transformants were selected on LB/carbenicillin (50 μ g/mL) plates. A single colony was used to inoculate 4 mL culture in a 15 mL culture tube. Strain ALS484(DE3) strain

10 carrying vector pET11a was used as a control. The cells were grown at 37°C and 250 rpm in an Innova 4230 Incubator Shaker (New Brunswick Scientific, Edison, NJ) for eight to nine hours. This culture (3 mL) was used to start an anaerobic fermentation. Two 100 mL anaerobic cultures of ALS(DE3)/pET11a and ALS(DE3)pEIITHrEI were grown in serum bottles using LB media supplemented with 0.4% glucose, 50 μ g/mL

15 carbenicillin, and 100 mM MOPS. The cultures were grown overnight at 37°C without shaking. The overnight grown cultures were sub-cultured in serum bottles using fresh LB media supplemented with 0.4% glucose, 50 μ g/mL carbenicillin, and 100 mM MOPS. The starting OD(600) of these cultures was adjusted to 0.3. These serum bottles were incubated at 37°C without shaking. After one hour of incubation, the cultures were

20 induced with 100 μ M IPTG. A 3 mL sample was taken from each of the serum bottles at 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, and 24 hours. The samples were transferred into two properly labeled 2 mL microcentrifuge tubes, each containing 1.5 mL sample. The samples were spun down in a microcentrifuge centrifuge at 14000 g for 3 minutes. The supernatant was passed through a 0.45 μ syringe filter, and the resulting

25 filtrate was stored at -20°C until further analysis. The formation of fermentation products, mainly lactate and 3-hydroxypropionate, was measured by detecting derivatized CoA esters of lactate and 3-HP using LC/MS.

The following methods were performed to convert lactate and 3-HP into their respective CoA esters. Briefly, the filtrates were mixed with CoA-reaction buffer (200

30 mM potassium phosphate buffer, 2 mM acetyl-CoA, and 0.1 mg/mL purified transferase) in 1:1 ratio. The reaction was allowed to proceed for 20 minutes at room temperature.

The reaction was stopped by adding 1 volume of 10% TFA. The sample was purified using Sep Pak Vac columns (Waters). The column was conditioned with methanol and washed two times with 0.1% TFA. The sample was then applied to the column, and the column was washed two more times with 0.1% TFA. The sample was eluted with 40% acetonitrile, 0.1% TFA. The acetonitrile was removed from the sample by vacuum centrifugation. The samples were then analyzed by LC/MS.

Analysis of the standard CoA/CoA thioester mixtures and the CoA thioester mixtures derived from fermentation broths were carried out using a Waters/Micromass ZQ LC/MS instrument which had a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and the single quadrupole mass spectrometer. LC separations were made using a 4.6 x 150 mm YMC ODS-AQ (3 μ m particles, 120 Å pores) reversed-phase chromatography column at room temperature. Two gradient elution systems were developed using different mobile phases for the separation of the CoA esters. These two systems are summarized in Table 3. Elution system 1 was developed to provide the most rapid and efficient separation of the five-component CoA/CoA thioester mixture (CoA, acetyl-CoA, lactyl-CoA, acrylyl-CoA, and propionyl-CoA), while elution system 2 was developed to provide baseline separation of the structurally isomeric esters lactyl-CoA and 3HP-CoA in addition to separation of the remaining esters listed above. In all cases, the flow rate was 0.250 mL/minute, and photodiode array UV absorbance was monitored from 200 nm to 400 nm. All parameters of the electrospray MS system were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest and production of characteristic fragment ions. The following instrumental parameters were used for ESI-MS detection of CoA and organic acid-CoA thioesters in the positive ion mode: Capillary: 4.0 V; Cone: 56 V; Extractor: 1 V; RF lens: 0 V; Source temperature: 100°C; Desolvation temperature: 300°C; Desolvation gas: 500 L/hour; Cone gas: 40 L/hour; Low mass resolution: 13.0; High mass resolution: 14.5; Ion energy: 0.5; Multiplier: 650. Uncertainties for reported mass/charge ratios (m/z) and molecular masses are $\pm 0.01\%$. Table 3 provides a summary of gradient elution systems for the separation of organic acid-Coenzyme A thioesters.

Table 3

System	Buffer A	Buffer B	Gradient	
			Time	Percent B
1	25 mM ammonium acetate 0.5 % acetic acid	ACN 0.5 % acetic acid	0	10
			40	40
			42	100
			47	100
			50	10
2	25 mM ammonium acetate 10 mM TEA 0.5 % acetic acid	ACN 0.5 % acetic acid	0	10
			10	10
			45	60
			50	100
			53	100
			54	10

The following methods were used to separate the derivatized 3-hydroxypropionyl-CoA, which was formed from 3-HP, from 2-hydroxypropionyl-CoA (i.e., lactyl-CoA), which was formed from lactate. Because these structural isomers have identical masses and mass spectral fragmentation behavior, the separation and identification of these analytes in a mixture depends on their chromatographic separation. While elution system 1 provided excellent separation of the CoA thioesters tested (Figure 46), it was unable to resolve 3-HP-CoA and lactyl-CoA. An alternative LC elution system was developed using ammonium acetate and triethylamine (system 2; Table 3).

The ability of system 2 to separate 3-HP-CoA and lactyl-CoA was tested on a mixture of these two compounds. Comparing the results from a mixture of 3-HP-CoA and lactyl-CoA (Figure 47, Panel A) to the results from lactyl-CoA only (Figure 47, Panel B) revealed that system 2 can separate 3-HP-CoA and lactyl-CoA. The mass spectrum recorded under peak 1 (Figure 47, Panel A insert) was used to identify peak 1 as being a hydroxypropionyl-CoA thioester when compared to Figure 46, Panel C. In addition, comparison of Panels A and B of Figure 47 as well as the mass spectra results

corresponding to each peak revealed that peak 1 corresponds to 3-HP-CoA and peak 2 corresponds to lactyl-CoA.

System 2 was used to confirm that *E. coli* transfected with pEIITHrEI produced 3-HP in that 3-HP-CoA was detected. Specifically, an ion chromatogram for $m/z = 840$ in the analysis of a CoA transferase-treated fermentation broth aliquot collected from a culture of *E. coli* containing pEIITHrEI revealed the presence of 3-HP-CoA (Figure 48, Panel A). The CoA transferase-treated fermentation broth aliquot collected from a culture of *E. coli* lacking pEIITHrEI did not exhibit the peak corresponding to 3-HP-CoA (Figure 48, Panel B). Thus, these results indicate that the pEIITHrEI plasmid directs the expression of polypeptides having propionyl-CoA transferase activity, lactyl-CoA dehydratase activity, and acrylyl-CoA hydratase activity. These results also indicate that expression of these polypeptides leads to the formation of 3-HP.

**Example 9 – Cloning nucleic acid molecules that encode
a polypeptide having acetyl CoA carboxylase activity**

Polypeptides having acetyl-CoA carboxylase activity catalyze the first committed step of the fatty acid synthesis by carboxylation of acetyl-CoA to malonyl-CoA. Polypeptides having acetyl-CoA carboxylase activity are also responsible for providing malonyl-CoA for the biosynthesis of very-long-chain fatty acids required for proper cell function. Polypeptides having acetyl-CoA carboxylase activity can be biotin dependent enzymes in which the cofactor biotin is post-translationally attached to a specific lysine residue. The reaction catalyzed by such polypeptides consists of two discrete half reactions. In the first half reaction, biotin is carboxylated by biocarbonate in an ATP-dependent reaction to form carboxybiotin. In the second half reaction, the carboxyl group is transferred to acetyl-CoA to form malonyl-CoA.

Prokaryotic and eukaryotic polypeptides having acetyl-CoA carboxylase activity exist. The prokaryotic polypeptide is a multi-subunit enzyme (four subunits), where each of the subunits is encoded by a different nucleic acid sequence. For example, in *E. coli*, the following genes encode for the four subunits of acetyl-CoA carboxylase:

accA: Acetyl-coenzyme a-carboxylase carboxyl transferase subunit alpha
(GenBank® accession number M96394)

accB: Biotin carboxyl carrier protein (GenBank® accession number U18997)

accC: Biotin carboxylase (GenBank® accession number U18997)

accD: Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (GenBank® accession number M68934)

5 The eukaryotic polypeptide is a high molecular weight multi-functional enzyme encoded by a single gene. For example, in *Saccharomyces cerevisiae*, the acetyl-CoA carboxylase can have the sequence set forth in GenBank® accession number M92156.

 The prokaryotic type acetyl-CoA carboxylase from *E. coli* was overexpressed using T7 promoter vector pFN476 as described elsewhere (Davis *et al. J. Biol. Chem.*,
10 275:28593-28598 (2000)). The eukaryotic type acetyl-CoA carboxylase gene was amplified from *Saccharomyces cerevisiae* genomic DNA. Two primers were designed to amplify the *acc1* gene from in *S. cerevisiae* (*acc1F* 5'-
atagGCGGCCGCAGGAATGCTGTATGAGCGAAGAAAGCTTATT C-3', SEQ ID
NO: 138 where the bold is homologous sequence, the italics is a *Not* I site, the underline
15 is a RBS, and the lowercase is extra; and *acc1R* 5'-*atgctcgcatCTCGAGTAG-CTAAATTAAATTACATCAATAGTA-3'*, SEQ ID NO: 139 where the bold is
homologous sequence, the italics is a *Xho* I site, and the lowercase is extra). The following PCR mix is used to amplify *acc1* gene 10X *pfu* buffer (10 µL), dNTP (10mM;
2 µL), cDNA (2 µL), *acc1F* (100 µM; 1 µL), *acc1R* (100 µM; 1 µL), *pfu* enzyme (2.5
20 units/µL; 2 µL), and DI water (82 µL). The following protocol was used to amplify the *acc1* gene. After performing PCR, the PCR product was separated on a gel, and the band corresponding to *acc1* nucleic acid (about 6.7 Kb) was gel isolated using Qiagen gel isolation kit. The PCR fragment is digested with *Not* I and *Xho* I (New England BioLab) restriction enzymes. The digested PCR fragment is then ligated to pET30a which was
25 restricted with *Not* I and *Xho* I and dephosphorylated with SAP enzyme. The *E. coli* strain DH10B was transformed with 1 µL of the ligation mix, and the cells were recovered in 1 mL of SOC media. The transformed cells were selected on LB/kanamycin (50 µg/µL) plates. Eight single colonies are selected, and PCR was used to screen for the correct insert. The plasmid having correct insert was isolated using Qiagen Spin Mini
30 prep kit.

To obtain a polypeptide having acetyl-CoA carboxylase activity, the plasmid pMSD8 or pET30a/acc1 overexpressing *E. coli* or *S. cerevisiae* acetyl-CoA carboxylase was transformed into Tuner pLacI chemically competent cells (Novagen, Madison, WI). The transformed cells were selected on LB/chloramphenicol (25 µg/mL) plus carbencillin
5 (50 µg/mL) or kanamycin (50 µg/mL).

A crude extract of this strain can be prepared in the following manner. An overnight culture of Tuner pLacI with pMSD8 is subcultured into 200 mL (in one liter baffle culture flask) of fresh M9 media supplemented with 0.4% glucose, 1 µg/mL thiamine, 0.1% casamino acids, and 50 µg/mL carbencillin or 50 µg/mL kanamycin and
10 25 µg/mL chloramphenicol. The culture is grown at 37°C in a shaker with 250 rpm agitation until it reaches an optical density at 600 nm of about 0.6. IPTG is then added to a final concentration of 100 µM. The culture is then incubated for an additional 3 hours with shaking speed of 250 rpm at 37°C. Cells are harvested by centrifugation at 8000 x g and are washed one time with 0.85% NaCl. The cell pellet was resuspended in a minimal
15 volume of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, and 5% glycerol. The cells are lysed by passing them two times through a French Pressure cell at 1000 psig pressure. The cell debris was removed by centrifugation for 20 minutes at 30,000 x g.

The enzyme can be assayed using a method from Davis *et al.* (*J. Biol. Chem.*,
20 275:28593-28598 (2000)).

Example 10 – Cloning a nucleic acid molecule that encodes a polypeptide having malonyl-CoA reductase activity from *Chloroflexus aurantiacus*

A polypeptide having malonyl-CoA reductase activity was partially purified from
25 *Chloroflexus aurantiacus* and used to obtained amino acid micro-sequencing results. The amino acid sequencing results were used to identify and clone the nucleic acid that encodes a polypeptide having malonyl-CoA reductase activity.

Biomass required for protein purification was grown in B. Braun BIostat B fermenters (B. Braun Biotech International GmbH, Melsungen, Germany). A glass vessel
30 fitted with a water jacket for heating was used to grow the required biomass. The glass

vessel was connected to its own control unit. The liquid working volume was 4 L, and the fermenter was operated at 55°C with 75 rpm of agitation. Carbon dioxide was occasionally bubbled through the headspace of the fermenter to maintain anaerobic conditions. The pH of the cultures was monitored using a standard pH probe and was maintained between 8.0 and 8.3. The inoculum for the fermenters was grown in two 250 mL bottles in an Innova 4230 Incubator, Shaker (New Brunswick Scientific, Edison, NJ) at 55°C with interior lights. The fermenters were illuminated by three 65 W plant light reflector lamps (General Electric, Cleveland, OH). Each lamp was placed approximately 50 cm away from the glass vessel. The media used for the inoculum and the fermenter culture was as follows per liter: 0.07 g EDTA, 1 mL micronutrient solution, 1 mL FeCl₃ solution, 0.06 g CaSO₄·2 H₂O, 0.1 g MgSO₄·7 H₂O, 0.008 g NaCl, 0.075 g KCl, 0.103 g KNO₃, 0.68 g NaNO₃, 0.111 g Na₂HPO₄, 0.2 g NH₄Cl, 1 g yeast extract, 2.5 g casamino acid, 0.5 g Glycyl-Glycine, and 900 mL DI water. The micronutrient solution contained the following per liter: 0.5 mL H₂SO₄ (conc.), 2.28 g MnSO₄·7 H₂O, 0.5 g ZnSO₄·7 H₂O, 0.5 g H₃BO₃, 0.025 g CuSO₄·2 H₂O, 0.025 g Na₂MoO₄·2 H₂O, and 0.045 g CoCl₂·6 H₂O. The FeCl₃ solution contained 0.2905 g FeCl₃ per liter. After adjusting the pH of the media to 8.2 to 8.4, 0.75 g/L Na₂S·9H₂O was added, the pH was readjusted to 8.2 to 8.4, and the media was filter-sterilized through a 0.22 µ filter.

The fermenter was inoculated with 500 mL of grown culture. The fermentation was stopped, and the biomass was harvested after the cell density was about 0.5 to 0.6 units at 600 nm.

The cells were harvested by centrifugation at 5000 x g (Beckman JLA 8.1000 rotor) at 4°C, washed with 1 volume of ice cold 0.85% NaCl, and centrifuged again. The cell pellet was resuspended in 30 mL of ice cold 100 mM Tris-HCl (pH 7.8) buffer that was supplemented with 2 mM DTT, 5 mM MgCl₂, 0.4 mM PEFABLOC (Roche Molecular Biochemicals, Indianapolis, IN), 1% streptomycin sulfate, and 2 tablets of Complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). The cell suspension was lysed by passing the suspension, three times, through a 50 mL French Pressure Cell operated at 1600 psi (gauge reading). Cell debris was removed by centrifugation at 30,000 x g (Beckman JA 25.50 rotor). The crude extract was filtered prior to chromatography using a 0.2 µm HT Tuffryn membrane

syringe filter (Pall Corp., Ann Arbor, MI). The protein concentration of the crude extract was 29 mg/mL, which was determined using the BioRad Protein Assay according to the manufacturer's microassay protocol. Bovine gamma globulin was used for the standard curve determination. This assay was based on the Bradford dye-binding procedure
5 (Bradford, *Anal. Biochem.*, 72:248 (1976)).

Before starting the protein purification, the following assay was used to determine the activity of malonyl-CoA reductase in the crude extract. A 50 μ L aliquot of the cell extract (29 mg/mL) was added to 10 μ L 1M Tris-HCl (final concentration in assay 100 mM), 10 μ L 10 mM malonyl CoA (final concentration in assay 1 mM), 5.5 μ L 5.5 mM
10 NADPH (final concentration in assay 0.3 mM), and 24.5 μ L DI water in a 96 well UV transparent plate (Corning, NY). The enzyme activity was measured at 45°C using SpectraMAX Plus 96 well plate reader (Molecular devices Sunnyvale, CA). The activity of malonyl-CoA reductase was monitored by measuring the disappearance of NADPH at 340 nm wavelength. The crude extract exhibited malonyl-CoA reductase activity.

15 The 5 mL (total 145 mg) protein cell extract was diluted with 20 mL buffer A (20 mM ethanolamine (pH 9.0), 5 mM $MgCl_2$, 2 mM DTT). The chromatographic protein purification was conducted using a BioLogic protein purification system (BioRad Hercules, CA). The 25 mL of cell suspension was loaded onto a UNO Q-6 ion-exchange column that had been equilibrated with buffer A at a rate of 1 mL/minute. After sample
20 loading, the column was washed with 2.5 times column volume of buffer A at a rate of 2 mL/minute. The proteins were eluted with a linear gradient of NaCl in buffer A from 0-0.33 M in 25 Column volume. During the entire chromatographic separation, three mL fractions were collected. The collection tubes contained 50 μ L of Tris-HCl (pH 6.5) so that the pH of the eluted sample dropped to about pH 7. Major chromatographic peaks
25 were detected in the region that corresponded to fractions 18 to 21 and 23 to 30. A 200 μ L sample was taken from these fractions and concentrated in a microcentrifuge at 4°C using a Microcon YM-10 columns (Millipore Corp., Bedford, MA) as per manufacture's instructions. To each of the concentrated fraction, buffer A-Tris (100 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 2 mM DTT) was added to bring the total volume to 100 μ L. Each of
30 these fractions was tested for the malonyl-CoA reductase activity using the spectrophotometric assay described above. The majority of specific malonyl CoA activity

was found in fractions 18 to 21. These fractions were pooled together, and the pooled sample was desalted using PD-10 column (Amersham Pharmacia Piscataway, NJ) as per manufacture's instructions.

5 The 10.5 mL of desalted protein extract was diluted with buffer A-Tris to a volume of 25 mL. This desalted diluted sample was applied to a 1 mL HiTrap Blue column (Amersham Pharmacia Piscataway, NJ) which was equilibrated with buffer A-Tris. The sample was loaded at a rate of 0.1 mL/minute. Unbound proteins were washed with 2.5 CV buffer A-Tris. The protein was eluted with 100 mM Tris (pH 7.8), 5 mM MgCl₂, 2 mM DTT, 2mM NADPH, and 1 M NaCl. During this separation process, one
10 mL fractions were collected. A 200 µL sample was drawn from fractions 49 to 54 and concentrated. Buffer A-Tris was added to each of the concentrated fractions to bring the total volume to 100 µL. Fractions were assayed for enzyme activity as described above. The highest specific activity was observed in fraction 51. The entire fraction 51 was concentrated as described above, and the concentrated sample was separated on an SDS-
15 PAGE gel.

Electrophoresis was carried out using a Bio-Rad Protean II minigel system and pre-cast SDS-PAGE gels (4-15%), or a Protean II XI system and 16 cm x 20 cm x 1mm SDS-PAGE gels (10%) cast as per the manufacturer's protocol. The gels were run according to the manufacturer's instructions with a running buffer of 25 mM Tris-HCl
20 (pH 8.3), 192 mM glycine, and 0.1% SDS.

A gel thickness of 1 mm was used to run samples from fraction 51. Protein from fraction 51 was loaded onto 10% SDS-PAGE (3 lanes, each containing 75 µg of total protein). The gels were stained briefly with Coomassie blue (Bio-Rad, Hercules, CA) and then destained to a clear background with a 10% acetic acid and 20% methanol solution.
25 The staining revealed a band of about 130 to 140 KDa.

The protein band of about 130-140 KDa was excised with no excess unstained gel present. An equal area gel without protein was excised as a negative control. The gel slices were placed in uncolored microcentrifuge tubes, prewashed with 50% acetonitrile in HPLC-grade water, washed twice with 50% acetonitrile, and shipped on dry ice to
30 Harvard Microchemistry Sequencing Facility, Cambridge, MA.

After *in-situ* enzymatic digestion of the polypeptide sample with trypsin, the resulting polypeptides were separated by micro-capillary reverse-phase HPLC. The HPLC was directly coupled to the nano-electrospray ionization source of a Finnigan LCQ quadrupole ion trap mass spectrometer (μ LC/MS/MS). Individual sequence spectra (MS/MS) were acquired on-line at high sensitivity for the multiple polypeptides separated during the chromatographic run. The MS/MS spectra of the polypeptides were correlated with known sequences using the algorithm Sequest developed at the University of Washington (Eng *et al.*, *J. Am. Soc. Mass Spectrom.*, 5:976 (1994)) and programs developed at Harvard (Chittum *et al.*, *Biochemistry*, 37:10866 (1998)). The results were reviewed for consensus with known proteins and for manual confirmation of fidelity.

A similar purification procedure was used to obtain another sample (protein 1 sample) that was subjected to the same analysis that was used to evaluate the fraction 51 sample.

The polypeptide sequence results indicated that the polypeptides obtained from both the fraction 51 sample and the protein 1 sample had similarity to the six (764, 799, 859, 923, 1090, 1024) contigs sequenced from the *C. aurantiacus* genome and presented on the Joint Genome Institute's web site (<http://www.jgi.doe.gov/>). The 764 contig was the most prominent of the six with about 40 peptide sequences showing similarity. BLASTX analysis of each of these contigs on the GenBank web site (<http://www.ncbi.nlm.nih.gov/BLAST/>) indicated that the DNA sequence of the 764 contig (4201 bases) encoded for polypeptides that had a dehydrogenase/reductase type activity. Close inspection of the 764 contig, however, revealed that this contig did not have an appropriate ORF that would encode for a 130-140 KDa polypeptide.

BASLTX analysis also was conducted using the other five contigs. The results of this analysis were as follows. The 799 contig (3173 bases) appeared to encode polypeptides having phosphate and dehydrogenase type activities. The 859 contig (5865 bases) appeared to encode polypeptides having synthetase type activities. The 923 contig (5660 bases) appeared to encode polypeptides having elongation factor and synthetase type activities. The 1090 contig (15201 bases) appeared to encode polypeptides having dehydrogenase/reductase and cytochrome and sigma factor activities. The 1024 contig (12276 bases) appeared to encode polypeptides having dehydrogenase and decarboxylase

and synthetase type activities. Thus, the 859 and 923 contigs were eliminated from any further analysis.

The results from the BLASTX analysis also revealed that the dehydrogenase found in the 1024 contig was most likely an inositol monophosphate dehydrogenase.

- 5 Thus, the 1024 contig was eliminated as a possible candidate that might encode for a polypeptide having malonyl-CoA reductase activity. The 799 contig also was eliminated since this contig is part of the OS17 polypeptide described above.

- This narrowed down the search to 2 contigs, the 764 and 1090 contigs. Since the contigs were identified using the same protein sample and the dehydrogenase activities
10 found in these contigs gave very similar BLASTX results, it was hypothesized that they are part of the same polypeptide. Additional evidence supporting this hypothesis was obtained from the discovery that the 764 and 1090 contigs are adjacent to each other in the *C. aurantiacus* genome as revealed by an analysis of scaffold data provided by the Joint Genome Institute. Sequence similarity and assembly analysis, however, revealed no
15 overlapping sequence between these two contigs, possibly due to the presence of gaps in the genome sequencing.

- The polypeptide sequences that belonged to the 764 and 1090 contigs were mapped. Based on this analysis, an appropriate coding frame and potential start and stop codons were identified. The following PCR primers were designed to PCR amplify a
20 fragment that encoded for a polypeptide having malonyl-CoA reductase activity: PRO140F 5'-ATGGCGACGGGCGAGTCCATGAG-3', SEQ ID NO:153; PRO140R 5'-GGACACGAAGAACAGGGCGACAC-3', SEQ ID NO:154; and PRO140UP 5'-GAACTGTCTGGAGTAAGGCTGTC-3', SEQ ID NO:155. The PRO140F primer was designed based on the sequence of the 1090 contig and corresponds to the start of the
25 potential start codon. The twelfth base was change from G to C to avoid primer-dimer formation. This change does not change the amino acid that was encoded by the codon. The PRO140R primer was designed based on sequence of the 764 contig and corresponds to a region located about 1 kB downstream from the potential stop codon. The PRO140UPF primer was designed based on sequence of the 1090 contig and corresponds
30 to a region located about 300 bases upstream of potential start codon.

Genomic *C. aurantiacus* DNA was obtained. Briefly, *C. aurantiacus* was grown in 50 mL cultures for 3 to 4 days. Cells were pelleted and washed with 5 mL of a 10 mM Tris solution. The genomic DNA was then isolated using the gram positive bacteria protocol provided with Gentra Genomic "Puregene" DNA isolation kit (Gentra Systems, Minneapolis, MN). The cell pellet was resuspended in 1 mL Gentra Cell Suspension Solution to which 14.2 mg of lysozyme and 4 μ L of 20 mg/mL proteinase K solution was added. The cell suspension was incubated at 37°C for 30 minutes. The precipitated genomic DNA was recovered by centrifugation at 3500g for 25 minutes and air-dried for 10 minutes. The genomic DNA was suspended in an appropriate amount of a 10 mM Tris solution and stored at 4°C.

Two PCR reactions were set-up using *C. aurantiacus* genomic DNA as template as follows:

PCR Reaction #1			PCR program
15	3.3 X <i>rTH</i> polymerase Buffer	30 μ L	94°C 2 minutes
	Mg(OAC) (25 mM)	4 μ L	29 cycles of:
	dNTP Mix (10 mM)	3 μ L	94°C 30 seconds
	PRO140F (100 μ M)	2 μ L	63°C 45 seconds
	PRO140R (100 μ M)	2 μ L	68°C 4.5 minutes
20	Genomic DNA (100 ng/mL)	1 μ L	68°C 7 minutes
	<i>rTH</i> polymerase (2 U/ μ L)	2 μ L	4°C Until further use
	<i>pfu</i> polymerase (2.5 U/ μ L)	0.25 μ L	
	DI water	55.75 μ L	
	Total	100 μ L	
25	PCR Reaction #2		PCR program
	3.3 X <i>rTH</i> polymerase Buffer	30 μ L	94°C 2 minutes
	Mg(OAC) (25 mM)	4 μ L	29 cycles of:
	dNTP Mix (10 mM)	3 μ L	94°C 30 seconds
30	PRO140UPF (100 μ M)	2 μ L	60°C 45 seconds
	PRO140R (100 μ M)	2 μ L	68°C 4.5 minutes

Genomic DNA (100 ng/mL)	1 μ L	68°C	7 minutes
<i>rTH</i> polymerase (2 U/ μ L)	2 μ L	4°C	Until further use
<i>pfu</i> polymerase 2.5 U/ μ L)	0.25 μ L		
DI water	55.75 μ L		
5 Total	100 μ L		

The products from both PCR reactions were separated on a 0.8% TAE gel. Both PCR reactions produced a product of 4.7 to 5 Kb in size. This approximately matched the expected size of a nucleic acid molecule that could encode a polypeptide having malonyl-CoA reductase activity.

Both PCR products were sequenced using sequencing primers (1090Fseq 5'-GATTCCGTATGTCACCCCTA-3', SEQ ID NO:156; and 764Rseq 5'-CAGGCGACTGGCAATCACAA-3', SEQ ID NO:157). The sequence analysis revealed a gap between the 764 and 1090 contigs. The nucleic acid sequence between the sequences from the 764 and 1090 contigs was greater than 300 base pairs in length (Figure 51). In addition, the sequence analysis revealed an ORF of 3678 bases that showed similarities to dehydrogenase/reductase type enzymes (Figure 52). The amino acid sequence encoded by this ORF is 1225 amino acids in length (Figure 50). Also, BLASTP analysis of the amino acid sequence encoded by this ORF revealed two short chain dehydrogenase domains (adh type). These results are consistent with a polypeptide having malonyl-CoA reductase activity since such an enzyme involves two reduction steps for the conversion of malonyl CoA to 3-HP. Further, the computed MW of the polypeptide was determined to be about 134 KDa.

PCR was conducted using the PRO140F/PRO140R primer pair, *C. aurantiacus* genomic DNA, and the protocol described above as PCR reaction #1. After the PCR was completed, 0.25 U of *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN) was added to the PCR mix, which was then incubated at 72°C for 10 minutes. The PCR product was column purified using Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified PCR product was then TOPO cloned into expression vector pCRT7/CT as per manufacture's instructions (Invitrogen, Carlsbad, CA). TOP10 F' chemical competent cells were transformed with the TOPO ligation mix as per

manufacture's instructions (Invitrogen, Carlsbad, CA). The cells were recovered for half an hour, and the transformants were selected on LB/ampicillin (100 µg/mL) plates. Twenty single colonies were selected, and the plasmid DNA was isolated using Qiagen spin Mini prep kit (Qiagen Inc., Valencia, CA).

- 5 Each of these twenty clones were tested for correct orientation and right insert size by PCR. Briefly, plasmid DNA was used as a template, and the following two primers were used in the PCR amplification: PCRT7 5'-GAGACCACAACGGTTTCCCTCTA-3', SEQ ID NO:158; and PRO140R 5'-GGACACGAAGAACAGGGCGACAC-3', SEQ ID NO:159. The following PCR reaction mix and program was used:

10	PCR Reaction	PCR program
	3.3 X <i>rTH</i> polymerase Buffer	7.5 µL -- 94°C 2 minutes
	Mg(OAC) (25 mM)	1 µL - 25 cycles of:
15	dNTP Mix (10 mM)	0.5 µL 94°C 30 seconds
	PCRT7 (100 µM)	0.125 µL 55°C 45 seconds
	PRO140R (100 µM)	0.125 µL 68°C 4 minutes
	Plasmid DNA	0.5 µL 68°C 7 minutes
	<i>rTH</i> polymerase (2 U/µL)	0.5 µL 4°C Until further use
20	DI water	14.75 µL
	Total	25 µL

- Out of twenty clone tested, only one clone exhibited the correct insert (Clone # P-10). Chemical competent cells of BL21(DE3)pLysS (Invitrogen, Carlsbad, CA) were transformed with 2 µL of the P-10 plasmid DNA as per the manufacture's instructions. 25 The cells were recovered at 37°C for 30 minutes and were plated on LB ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL).

- A 20 mL culture of BL21(DE3)pLysS/P-10 and a 20 mL control culture of BL21(DE3)pLysS was incubated overnight. Using the overnight cultures as an inoculum, 30 two 100 mL BL21(DE3)pLysS/P-10 clone cultures and two control strain cultures (BL21(DE3)pLysS) were started. All the cultures were induced with IPTG when they

reached an OD of about 0.5 at 600 nm. The control strain culture was induced with 10 μ M IPTG or 100 μ M IPTG, while one of the BL21(DE3)pLysS/P-10 clone cultures was induced with 10 μ M IPTG and the other with 100 μ M IPTG. The cultures were grown for 2.5 hours after induction. Aliquots were taken from each of the culture flasks before and
5 after 2.5 hours of induction and separated using 4-15% SDS-PAGE to analyze polypeptide expression. In the induced BL21(DE3)pLysS/P-10 samples, a band corresponding to a polypeptide having a molecular weight of about 135 KDa was observed. This band was absent in the control strain samples and in samples taken before IPTG induction.

10 To assess malonyl-CoA reductase activity, BL21(DE3)pLysS/P-10 and BL21(DE3)pLysS cells were cultured and then harvested by centrifugation at 8,000 x g (Rotor JA 16.250, Beckman Coulter, Fullerton, CA). Once harvested, the cells were washed once with an equal volume of a 0.85% NaCl solution. The cell pellets were resuspended into 100 mM Tris-HCl buffer that was supplemented with 5 mM Mg_2Cl and
15 2 mM DTT. The cells were disrupted by passing twice through a French Press Cell at 1,000 psi pressure (Gauge value). The cell debris was removed by centrifugation at 30,000 x g (Rotor JA 25.50, Beckman Coulter, Fullerton, CA). The cell extract was maintained at 4°C or on ice until further use.

Activity of malonyl-CoA reductase was measured at 37°C for both the control
20 cells and the IPTG-induced cells. The activity of malonyl-CoA reductase was monitored by observing the disappearance of added NADPH as described above. No activity was found in the cell extract of the control strain, while the cell extract from the IPTG-induced BL21(DE3)pLysS/P-10 cells displayed malonyl-CoA reductase activity with a specific activity calculated to be about 0.0942 μ mole/minute/mg of total protein.

25 Malonyl-CoA reductase activity also was measured by analyzing 3-HP formation from malonyl CoA using the following reaction conducted at 37°C:

	Volume	Final conc.
Tris HCl (1M)	10 μ L	100mM
Malonyl CoA (10mM)	40 μ L	4 mM
30 NADPH (10 mM)	30 μ L	3 mM
Cell-extract	20 μ L	

Total 100 μ L

The reaction was carried out at 37°C for 30 minutes. In the control reaction, a cell extract from BL21(DE3)pLysS was added to a final concentration of 322 mg total protein. In the experimental reaction mix, a cell extract from BL21(DE3)pLysS/P-10 was added to a final concentration of 226 mg of total protein. The reaction mixtures were frozen at -20°C until further analysis.

Chromatographic separation of the components in the reaction mixtures was performed using a HPX-87H (7.8x300mm) organic acid HPLC column (BioRad Laboratories, Hercules, CA). The column was maintained at 60°C. Mobile phase composition was HPLC grade water pH to 2.5 using trifluoroacetic acid (TFA) and was delivered at a flow rate of 0.6 mL/minute.

Detection of 3-HP in the reaction samples was accomplished using a Waters/Micromass ZQ LC/MS instrument consisting of a Waters 2690 liquid chromatograph (Waters Corp., Milford, MA) with a Waters 996 Photo-diode Array (PDA) absorbance monitor placed in series between the chromatograph and the single quadrupole mass spectrometer. The ionization source was an Atmospheric Pressure Chemical Ionization (APCI) ionization source. All parameters of the APCI-MS system were optimized and selected based on the generation of the protonated molecular ion $([M+H])^+$ of 3-HP. The following parameters were used to detect 3-HP in the positive ion mode: Corona: 10 μ A; Cone: 20V; Extractor: 2V; RF lens: 0.2V; Source temperature: 100°C; APCI-Probe temperature: 300°C; Desolvation gas: 500L/hour; Cone gas: 50L/hour; Low mass resolution: 15; High mass resolution: 15; Ion energy: 1.0; Multiplier: 650. Data was collected in Selected Ion Reporting (SIR) mode set at $m/z = 90.9$.

Both the control reaction sample and the experimental reaction sample were probed for presence of 3-HP using the HPLC-mass spectroscopy technique. In the control samples, no 3-HP peak was observed, while the experimental sample exhibited a peak that matched the retention and the mass of 3-HP.

30

Example 11 – Constructing recombinant cells that produce 3-HP

A pathway to make 3-hydroxypropionate directly from glucose via acetyl CoA is presented in Figure 44. Most organisms such as *E. coli*, *Bacillus*, and yeast produce acetyl CoA from glucose via glycolysis and the action of pyruvate dehydrogenase. In order to divert the acetyl CoA generated from glucose, it is desirable to overexpress two genes, one encoding for acetyl CoA carboxylase and the other encoding malonyl-CoA reductase. As an example, these genes are expressed in *E. coli* through a T7 promoter using vectors pET30a and pFN476. The vector pET30a has a pBR *ori* and kanamycin resistance, while pFN476 has pSC101 *ori* and uses carbencillin resistance for selection. Because these two vectors have compatible *ori* and different markers they can be maintained in *E. coli* at the same time. Hence, the constructs used to engineer *E. coli* for direct production of 3-hydroxypropionate from glucose are pMSD8 (pFN476/accABCD) (Davis *et al.*, *J. Biol. Chem.*, 275:28593-28598, 2000) and pET30a/malonyl-CoA reductase or pET30a/*acc1* and pFN476/malonyl-CoA reductase. The constructs are depicted in Figure 45.

To test the production of 3-hydroxypropionate from glucose, *E. coli* strain Tuner pLacI carrying plasmid pMSD8 (pFN476/accABCD) and pET30a/malonyl-CoA reductase or pET30a/*acc1* and pFN476/malonyl-CoA reductase are grown in a B. Braun BIOSTAT B fermenter. A glass vessel fitted with a water jacket for heating is used to conduct this experiment. The fermenter working volume is 1.5 L and is operated at 37°C. The fermenter is continuously supplied with oxygen by bubbling sterile air through it at a rate of 1 vvm. The agitation is cascaded to the dissolve oxygen concentration which is maintained at 40% DO. The pH of the liquid media is maintained at 7 using 2 N NaOH. The *E. coli* strain is grown in M9 media supplemented with 1% glucose, 1 µg/mL thiamine, 0.1% casamino acids, 10 µg/mL biotin, 50 µg/mL carbencillin, 50 µg/mL kanamycin, and 25 µg/mL chloramphenicol. The expression of the genes is induced when the cell density reached 0.5 OD(600nm) by adding 100 µM IPTG. After induction, samples of 2 mL volume are taken at 1, 2, 3, 4, and 8 hours. In addition, at 3 hours after induction, a 200 mL sample is taken to make a cell extract. The 2 mL samples are spun, and the supernatant is used to analyze products using LC/MS technique. The supernatant is stored at -20°C until further analysis.

The extract is prepared by spinning the 200 mL of cell suspension at 8000 g and washing the cell pellet with of 50 mL of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, and 5% glycerol. The cell suspension is spun again at 8000 g, and the pellet is resuspended into 5 mL of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, and 5% glycerol. The cells are disrupted by passing twice through a French Press at 1000 psg. The cell debris is removed by centrifugation for 20 minutes at 30,000 g. All the operations are conducted at 4°C. To demonstrated *in vitro* formation of 3-hydroxypropionate using this recombinant cell extract, the following reaction of 200 µL is conducted at 37°C. The reaction mix is as follows: Tris HCl (pH 8.0; 100 mM), ATP (1 mM), MgCl₂ (5 mM), KCl (100 mM), DTT (5 mM), NaHCO₃ (40 mM), NADPH (0.5 mM), acetyl CoA (0.5 mM), and cell extract (0.2 mg). The reaction is stopped after 15 minutes by adding 1 volume of 10% trifluoroacetic acid (TFA). The products of this reaction are detected using an LC/MS technique.

The detection and analysis for the presence of 3-hydroxypropionate in the supernatant and the *in vitro* reaction mixture is carried out using a Waters/Micromass ZQ LC/MS instrument. This instrument consists of a Waters 2690 liquid chromatograph with a Waters 2410 refractive index detector placed in series between the chromatograph and the single quadropole mass spectrometer. LC separations are made using a Bio-Rad Aminex 87-H ion-exchange column at 45°C. Sugars, alcohol, and organic acid products are eluted with 0.015% TFA buffer. For elution, the buffer is passed at a flow rate of 0.6 mL/minute. For detection and quantification of 3-hydroxypropionate, a sample obtained from TCI, America (Portland, OR) is used as a standard.

Example 12 Cloning of propionyl-CoA transferase, lactyl-CoA dehydratase (LDH), and a hydratase (OS19) for Expression in *Saccharomyces cerevisiae*

The pESC Yeast Epitope Tagging Vector System (Stratagene, La Jolla, CA) was used in cloning the genes involved in 3-hydroxypropionic acid production via lactic acid. The pESC vectors each contain GAL1 and GAL10 promoters in opposing directions, allowing the expression of two genes from each vector. The GAL1 and GAL10 promoters are repressed by glucose and induced by galactose. Each of the four available pESC vectors has a different yeast-selectable marker (HIS3, TRP1, LEU2, URA3) so

- multiple plasmids can be maintained in a single strain. Each cloning region has a polylinker site for gene insertion, a transcription terminator, and an epitope coding sequence for C-terminal or N-terminal epitope tagging of expressed proteins. The pESC vectors also have a ColE1 origin of replication and an ampicillin resistance gene to allow
- 5 replication and selection in *E. coli*. The following vector/promoter/nucleic acid combinations were constructed:

Vector	Promoter	Polypeptide	Source of nucleic acid
pESC-Trp	GAL1	OS19 hydratase	<i>Chloroflexus aurantiacus</i>
	GAL10	E1	<i>Megasphaera elsdenii</i>
pESC-Leu	GAL1	E2 α	<i>Megasphaera elsdenii</i>
	GAL10	E2 β	<i>Megasphaera elsdenii</i>
pESC-His	GAL1	D-LDH	<i>Escherichia coli</i>
	GAL10	PCT	<i>Megasphaera elsdenii</i>

The primers used were as follows:

- 10 OS19APAF: 5'-ATAGGGCCCAGGAGATCAAACCATGGGTGAAGAGTCT-
CTGGTTC-3' (SEQ ID NO:164)
- OS19SALR: 5'-CCTCTGCTACAGTCGACACAACGACCACTGAAGTTG-
GGAG-3' (SEQ ID NO:165)
- OS19KPNR: 5'-AGTCTGCTATCGGTACCTCAACGACCACTGAAGTTG-
15 GGAG-3' (SEQ ID NO:166)
- ENOTF: 5'-ATAGCGGCCGCATAATGGATACTCTCGGAATCGACG-
TTGG-3' (SEQ ID NO:167)
- EICLAR: 5'-CCCCATCGATACATATTTCTTGATTTTATCATAAGCA-
ATC-3' (SEQ ID NO:168)
- 20 EII α APAF: 5'-CCAGGGCCCATAATGGGTGAAGAAAAACAGTAGA-
TATTG-3' (SEQ ID NO:169)
- EII α SALR: 5'-GGTAGACTTGTCGACGTAGTGGTTTCCTCCTTCATT-
GG-3' (SEQ ID NO:170)
- EII β NOTF: 5'-ATAGCGGCCGCATAATGGGTCAGATCGACGAACCTTA-

TCAG-3'(SEQ ID NO:171)

EII β SPER: 5'-AGGTTCAACTAGTTCGTAGAGGATTTCCGAGAAAGC-
CTG-3'(SEQ ID NO:172)

LDHAPAF: 5'-CTAGGGCCCCATAATGGAACCTCGCCGTTTATAG-

5 CAC-3'(SEQ ID NO:173)

LDHXHOR: 5'-ACTTCTCGAGTTAAACCAGTTCGTTCTGGGCA-
GGT-3'(SEQ ID NO:174)

PCTSPEF: 5'-GGGACTAGTATAATGGGAAAAGTAGAAATCAT-
TACAG-3'(SEQ ID NO:175)

10 PCTPACR: 5'-CGGCTTAATTAACAGCAGAGATTTATTTTTC-
GTCC-3'(SEQ ID NO:176)

All restriction enzymes were obtained from New England Biolabs, Beverly, MA. All plasmid DNA preparations were done using QIAprep Spin Miniprep Kits, and all gel purifications were done using QIAquick Gel Extraction Kits (Qiagen, Valencia, CA).

15

A. Construction of the pESC-Trp/OS19 hydratase vector

Two constructs in pESC-Trp were made for the OS19 nucleic acid from *C. aurantiacus*. One of these constructs utilized the *Apa* I and *Sal* I restriction sites of the GAL1 multiple cloning site and was designed to include the c-myc epitope. The second
20 construct utilized the *Apa* I and *Kpn* I sites and thus did not include the c-myc epitope sequence.

Six μ g of pESC-Trp vector DNA was digested with the restriction enzyme *Apa* I and the digest was purified using a QIAquick PCR Purification Column. Three μ g of the *Apa* I-digested vector DNA was then digested with the restriction enzyme *Kpn* I, and 3 μ g
25 was digested with *Sal* I. The double-digested vector DNAs were separated on a 1% TAE-agarose gel, purified, dephosphorylated with shrimp alkaline phosphatase (Roche Biochemical Products, Indianapolis, IN), and purified with a QIAquick PCR Purification Column.

The nucleic acid encoding the *Chloroflexus aurantiacus* polypeptide having
30 hydratase activity (OS19) was amplified from genomic DNA using the PCR primer pair OS19APAF and OS19SALR and the primer pair OS19APAF and OS19KPNR.

OS19APAF was designed to introduce an *Apa* I restriction site and a translation initiation site (ACCATGG) at the beginning of the amplified fragment. The OS19KPNR primer was designed to introduce a *Kpn* I restriction site at the end of the amplified fragment and to contain the translational stop codon for the hydratase gene. OS19SALR introduces a *Sal* I site at the end of the amplified fragment and has an altered stop codon so that translation continues in-frame through the vector c-myc epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *C. aurantiacus* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase (Roche) in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 2.25 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 1 minute, and 72°C for 2.25 minutes; and a final extension for 7 minutes at 72°C. The amplification product was then separated by gel electrophoresis using a 1% TAE-agarose gel. A 0.8 Kb fragment was excised from the gel and purified for each primer pair. The purified fragments were digested with *Kpn* I or *Sal* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Apa* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

50-60 ng of the digested PCR product containing the nucleic acid encoding the *C. aurantiacus* polypeptide having hydratase activity (OS19) and 50 ng of the prepared pESC-Trp vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LB plates containing 100 μ g/mL of carbenicillin (LBC). Individual colonies were screened using colony PCR with the appropriate PCR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells was used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the nucleic acid from genomic DNA.

Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR. A construct with a confirmed sequence was transformed into *S. cerevisiae* strain YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). Transformation reactions were plated on SC-Trp media (see Stratagene pESC Vector Instruction Manual for media recipes). Individual yeast colonies were screened for the presence of the OS19 nucleic acid by colony PCR. Colonies were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 5 units of zymolase and heated at 37°C for 10 minutes. Three µL of this suspension was then used in a 25 µL PCR reaction using the PCR reaction mixture and program described for the colony screen of the *E. coli* transformants. The pESC-Trp vector was also transformed into YPH500 for use as a hydratase assay control and transformants were screened by PCR using GAL1 and GAL10 primers.

B. Construction of the pESC-Trp/OS19/EI hydratase vector

Plasmid DNA of a pESC-Trp/OS19 construct (*Apa* I-*Sal* I sites) with confirmed sequence and positive assay results was used for insertion of the nucleic acid for the *M. elsdenii* E1 activator polypeptide downstream of the GAL10 promoter. Three µg of plasmid DNA was digested with the restriction enzyme *Cla* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Not* I, and the digest was inactivated by heating to 65°C for 20 minutes. The double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche), separated on a 1% TAE-agarose gel, and gel purified.

The nucleic acid encoding the *M. elsdenii* E1 activator polypeptide was amplified from genomic DNA using the PCR primer pair EINOTF and EICLAR. EINOTF was designed to introduce a *Not* I restriction site and a translation initiation site at the beginning of the amplified fragment. The EICLAR primer was designed to introduce a *Cla* I restriction site at the end of the amplified fragment and to contain an altered translational stop codon to allow in-frame translation of the FLAG epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii* genomic DNA, 0.2 µM of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 µL. The PCR reaction was performed in an MJ Research PTC100

under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 3 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 3 minutes; and a final extension for 7 minutes at 72°C. The amplification product was then separated by gel electrophoresis using a 1% TAE-agarose gel, and a 0.8 Kb fragment was excised and purified. The
5 purified fragment was digested with *Cla* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Not* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

60 ng of the digested PCR product containing the nucleic acid for the *M. elsdenii*
10 E1 activator polypeptide and 70 ng of the prepared pESC-Trp/OS19 hydratase vector were ligated using T4 DNA ligase at 16°C for 16 hours. One µL of the ligation reaction was used to electroporate 40 µL of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC media. Individual colonies were screened using colony PCR with the EINOTF and EICLAR primers. Individual colonies were
15 suspended in about 25 µL of 10 mM Tris, and 2 µL of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 µL of the heated cells used in a 25 µL PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 µM each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as
20 described above for amplification of the gene from genomic DNA. Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR.

C. Construction of the pESC-Leu/EII α /EII β vector

25 Three µg of DNA of the vector pESC-Leu was digested with the restriction enzyme *Apa* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Sal* I, and the digest was inactivated by heating to 65°C for 20 minutes. The double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche), separated on a 1% TAE-
30 agarose gel, and gel purified.

The nucleic acid encoding the *M. elsdenii* E2 α polypeptide was amplified from genomic DNA using the PCR primer pair EII α APAF and EII α SALR. EII α APAF was designed to introduce an *Apa* I restriction site and a translation initiation site at the beginning of the amplified fragment. The EII α SALR primer was designed to introduce a *Sal* I restriction site at the end of the amplified fragment and to contain an altered translational stop codon to allow in-frame translation of the c-myc epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 3 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 1 minute, and 72°C for 3 minutes; and a final extension for 7 minutes at 72°C. The amplification product was then separated by gel electrophoresis using a 1% TAE-agarose-gel, and a 1.3 Kb fragment was excised and purified. The purified fragment was digested with *Apa* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Sal* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

80 ng of the digested PCR product containing the nucleic acid encoding the *M. elsdenii* E2 α polypeptide and 80 ng of the prepared pESC-Leu vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC media. Individual colonies were screened using colony PCR with the EII α APAF and EII α SALR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA. Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR.

Plasmid DNA of a pESC-Leu/EII α vector with confirmed sequence was used for insertion of the nucleic acid encoding the *M. elsdenii* E2 β polypeptide. Three μ g of plasmid DNA was digested with the restriction enzyme *Spe* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the
5 restriction enzyme *Not* I and gel purified from a 1% TAE-agarose gel. The double-digested vector DNA was then dephosphorylated with shrimp alkaline phosphatase (Roche) and purified with a QIAquick PCR Purification Column.

The nucleic acid encoding the *M. elsdenii* E2 β polypeptide was amplified from genomic DNA using the PCR primer pair EII β NOTF and EII β SPER. The EII β NOTF
10 primer was designed to introduce a *Not* I restriction site and a translation initiation site at the beginning of the amplified fragment. The EII β SPER primer was designed to introduce an *Spe* I restriction site at the end of the amplified fragment and to contain an altered translational stop codon to allow for in-frame translation of the FLAG epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii*
15 genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 3 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 3 minutes; and a final
20 extension for 7 minutes at 72°C. The amplification product was separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.1 Kb fragment was excised and purified. The purified fragment was digested with *Spe* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Not* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

25 38 ng of the digested PCR product containing the nucleic acid encoding the *M. elsdenii* E2 β polypeptide and 50 ng of the prepared pESC-Leu/EII α vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* ElectromaxTM DH10BTM cells. The electroporated cells were plated onto LBC plates. Individual colonies were screened using colony PCR with
30 the EII β NOTF and EII β SPER primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant

suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 µL of the heated cells was used in a 25 µL PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 µM each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above
5 for amplification of the gene from genomic DNA.

Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR. A pESC-Leu/EIIα/EIIβ construct with a confirmed sequence was co-transformed along with the pESC-Trp/OS19/EI vector into *S. cerevisiae* strain YPH500 using a Frozen-EZ Yeast
10 Transformation II™ Kit (Zymo Research, Orange, CA). Transformation reactions were plated on SC-Trp-Leu media. Individual yeast colonies were screened for the presence of the OS19, E1, E2α, and E2β nucleic acid by colony PCR. Colonies were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 5 units of zymolase and heated at 37°C for 10 minutes. ~~Three µL of this suspension was then used in a 25 µL PCR~~
15 reaction using the PCR reaction mixtures and programs described for the colony screens of the *E. coli* transformants. The pESC-Trp/OS19 and pESC-Leu vectors were also co-transformed into YPH500 for use as a lactyl-CoA dehydratase assay control. These transformants were colony screened using the GAL1 and GAL10 primers (Instruction manual, pESC-Yeast Epitope Tagging Vectors, Stratagene).

20

D. Construction of the pESC-His/D-LDH/PCT vector

Three µg of DNA of the vector pESC-His was digested with the restriction enzyme *Xho* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Apa* I and gel purified
25 from a 1% TAE-agarose gel. The double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche) and purified using a QIAquick PCR Purification Column.

The *E. coli* D-LDH gene was amplified from genomic DNA of strain DH10B using the PCR primer pair LDHAPAF and LDHXHR. LDHAPAF was designed to
30 introduce an *Apa* I restriction site and a translation initiation site at the beginning of the amplified fragment. The LDHXHR primer was designed to introduce an *Xho* I

restriction site at the end of the amplified fragment and to contain the translational stop codon for the D-LDH gene. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *E. coli* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 2 minutes; 24 cycles of 94°C for 30 seconds, 64°C for 45 seconds, and 72°C for 2 minutes; and a final extension for 7 minutes at 72°C. The amplification product was separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.0 Kb fragment was excised and purified. The purified fragment was digested with *Apa* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Xho* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

80 ng of the digested PCR product containing the *E. coli* D-LDH gene and 80 ng of the prepared pESC-His vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC media. Individual colonies were screened using colony PCR with the LDHAPAF and LDHXHR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA. Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR.

Plasmid DNA of a pESC-His/D-LDH construct with a confirmed sequence was used for insertion of the nucleic acid encoding the *M. elsdenii* PCT polypeptide. Three μ g of plasmid DNA was digested with the restriction enzyme *Pac* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Spe* I and gel purified from a 1% TAE-agarose gel. The

double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche) and purified with a QIAquick PCR Purification Column.

The nucleic acid encoding the *M. elsdenii* PCT polypeptide was amplified from genomic DNA using the PCR primer pair PCTSPEF and PCTPACR. PCTSPEF was
5 designed to introduce an *Spe* I restriction site and a translation initiation site at the beginning of the amplified fragment. The PCTPACR primer was designed to introduce a *Pac* I restriction site at the end of the amplified fragment and to contain the translational stop codon for the PCT gene. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP,
10 and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 56°C for 45 seconds, and 72°C for 2.5 minutes; 24 cycles of 94°C for 30 seconds, 64°C for 45 seconds, and 72°C for 2.5 minutes; and a final extension for 7 minutes at 72°C. The
15 amplification product was separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.55 Kb fragment was excised and purified. The purified fragment was digested with *Pac* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Spe* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.
20 95 ng of the digested PCR product containing the nucleic acid encoding the *M. elsdenii* PCT polypeptide and 75 ng of the prepared pESC-His/D-LDH vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC plates. Individual colonies were screened using colony PCR
25 with the PCTSPEF and PCTPACR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq
30 DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA.

Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR. A construct with a confirmed sequence was transformed into *S. cerevisiae* strain YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). Transformation reactions were plated on SC-His media. Individual yeast colonies were screened for the presence of the D-LDH and PCT genes by colony PCR. Colonies were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 5 units of zymolase and heated at 37°C for 10 minutes. Three µL of this suspension was then used in a 25 µL PCR reaction using the PCR reaction mixture and program described for the colony screen of the *E. coli* transformants. The pESC-His vector was also transformed into YPH500 for use as an assay control, and transformants were screened by PCR using GAL1 and GAL10 primers.

Example 13 - Expression of Enzymes in *S. cerevisiae*

A. Hydratase Activity in Transformed Yeast

Individual colonies carrying the pESC-Trp/OS19 construct or the pESC-Trp vector (negative control) were used to inoculate 5 mL cultures of SC-Trp media containing 2% glucose. These cultures were grown for 16 hours at 30°C and used to inoculate 35 mL of the same media. The subcultures were grown for 7 hours at 30°C, and their OD_{600s} were determined. A volume of cells giving an OD x volume equal to 40 was pelleted, washed with SC-Trp media with no carbon source, and repelleted. The cells were suspended in 5 mL of SC-Trp media containing 2% galactose and used to inoculate a total volume of 100 mL of this media. Cultures were grown for 17.5 hours at 30°C and 250 rpm. Cells were then pelleted, rinsed in 0.85% NaCl, and repelleted. Cell pellets (70 mg) were suspended in 140 µL of 50 mM TrisHCl, pH 7.5, and an equal volume (pellet plus buffer) of pre-rinsed glass beads (Sigma, 150-212 microns) was added. This mixture was vortexed for 30 seconds and placed on ice for 1 minute, and the vortexing/cooling cycle was repeated 8 additional times. The cells were then centrifuged for 6 minutes at 5,000g, and the supernatant was removed to a fresh tube. The beads/pellet were washed twice with 250 µL of buffer, centrifuged, and the supernatants joined with the first supernatant.

An *E. coli* strain carrying the pETBlue-1/OS19 construct, described previously, was used as a positive control for hydratase assays. A culture of this strain was grown to saturation overnight and diluted 1:20 the following morning in fresh LBC media. The culture was grown at 37°C and 250 rpm to an OD₆₀₀ of 0.6, at which point it was induced with IPTG at a final concentration of 1 mM. The culture was incubated for an additional two hours at 37°C and 250 rpm. Cells were pelleted, washed with 0.85 % NaCl, and repelleted. Cells were disrupted using BugBuster™ Protein Extraction Reagent and Benzonase® (Novagen) as per manufacturer's instructions with a 20 minute incubation at room temperature. After centrifugation at 16,000g and 4°C, the supernatant was transferred to a new tube and used in the activity assay.

Total protein content of cell extracts from *S. cerevisiae* described above were quantified using a microplate Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The OS19 constructs (both *Apa* I-*Sal* I and *Apa* I-*Kpn* I sites) in YPH500, the pESC-Trp negative control in YPH500, and the pETBlue-1/OS19-construct in *E. coli* were tested for their ability to convert acrylyl-CoA to 3-hydroxypropionyl-CoA. The assay was conducted as previously described for the pETBlue-1/OS19 constructs in the *E. coli* Tuner strain. When cell extract of the negative control strain was added to the reaction mixture containing acrylyl-CoA, one dominant peak of MW 823 was exhibited. This peak corresponds to acrylyl-CoA and indicates that acrylyl-CoA was not converted to any other product. When cell extract of the strain carrying a pESC-Trp/OS19 construct (either *Apa* I-*Sal* I or *Apa* I-*Kpn* I sites) was added to the reaction mix, the dominant peak shifted to MW 841, which corresponds to 3-hydroxypropionyl-CoA. The reaction mix from the *E. coli* control also showed the MW 841 peak. A time course study was conducted for the pESC-Trp/OS19(*Apa* I-*Sal* I) construct, which measured the appearance of the MW 841 and MW 823 peaks after 0, 1, 3, 7, 15, 30, and 60 minutes of reaction time. An increase in the 3-hydroxypropionyl-CoA peak was seen over time with the cell extracts from both this construct and the *E. coli* control, whereas cell extract from the YPH500 strain with vector only showed a dominant acrylyl-CoA peak.

B. Propionyl CoA-Transferase Activity in Transformed Yeast

Individual colonies of *S. cerevisiae* strain YPH500 carrying the pESC-His/D-LDH or pESC-His/D-LDH/PCT construct or the pESC-His vector with no insert (negative control) were used to inoculate 5 mL cultures of SC-His media containing 2% glucose.

- 5 These cultures were grown for 16 hours at 30°C and 250 rpm and were then used to inoculate 35 mL of the same media. The subcultures were grown for 7 hours at 30°C, and their OD₆₀₀s were determined. For each strain, a volume of cells giving an OD x volume equal to 40 was pelleted, washed with SC-His media with no carbon source, and repelleted. The cells were suspended in 5 mL of SC-His media containing 2% galactose
- 10 and used to inoculate a total volume of 100 mL of this media. Cultures were grown for 16.75 hours at 30°C and 250 rpm. Cells were then pelleted, rinsed in 0.85% NaCl, and repelleted. Cell pellets (70 mg) were suspended in 140 µL of 100 mM potassium phosphate buffer, pH 7.5, and an equal volume (pellet plus buffer) of pre-rinsed glass beads (Sigma, 150-212 microns) was added. This mixture was vortexed for 30 seconds
- 15 and placed on ice for 1 minute, and the vortexing/cooling cycle was repeated 8 additional times. The cells were then centrifuged for 6 minutes at 5,000g, and the supernatant was removed to a fresh tube. The beads/pellet were washed twice with 250 µL of buffer and centrifuged, and the supernatants joined with the first supernatant.

- An *E. coli* strain carrying the pETBlue-1/PCT construct, described previously,
- 20 was used as a positive control for propionyl CoA transferase assays. A culture of this strain was grown to saturation overnight and diluted 1:20 the following morning in fresh LB media containing 100 µg/mL of carbenicillin. The culture was grown at 37°C and 250 rpm to an OD₆₀₀ of 0.6, at which point it was induced with IPTG at a final concentration of 1 mM. The culture was incubated for an additional two hours at 37°C
- 25 and 250 rpm. Cells were pelleted, washed with 0.85 % NaCl, and repelleted. Cells were disrupted using BugBuster™ Protein Extraction Reagent and Benzonase® (Novagen) as per manufacturer's instructions with a 20 minute incubation at room temperature. After centrifugation at 16,000g and 4°C, the supernatant was transferred to a new tube and used in the activity assay.

- 30 Total protein content of cell extracts was quantified using a microplate Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The D-LDH and D-LDH/PCT constructs in *S.*

cerevisiae strain YPH500, the pESC-His negative control in YPH500, and the pETBlue-1/PCT construct in *E. coli* were tested for their ability to catalyze the conversion of propionyl-CoA and acetate to acetyl-CoA and propionate. The assay mixture used was that previously described for the pETBlue-1/PCT constructs in the *E. coli* Tuner strain.

- 5 When 1 μ g of total cell extract protein of the negative control strain or the YPH500/pESC-His/D-LDH strain was added to the reaction mixture, no increase in absorbance (0.00 to 0.00) was seen over 11 minutes. Increases in absorbance from 0.00 to 0.04 and from 0.00 to 0.06 were seen, respectively, with 1 μ g of cell extract protein from the YPH500/pESC-His/D-LDH/PCT strain and the *E. coli*/PCT strain. With 2 mg
10 of total cell extract protein, the negative control strain and the YPH500/pESC-His/D-LDH strain showed an increase in absorbance from 0.00 to 0.01 over 11 minutes, whereas increases from 0.00 to 0.10 and 0.00 to 0.08 were seen, respectively, with the YPH500/pESC-His/D-LDH/PCT strain and the *E. coli*/PCT strain.

15 C. Lactyl-CoA Dehydratase Activity in Transformed Yeast

- Individual colonies of *S. cerevisiae* strain YPH500 carrying the pESC-His/D-LDH or pESC-His/D-LDH/PCT construct or the pESC-His vector with no insert (negative control) were used to inoculate 5 mL cultures of SC-His media containing 4% glucose. These cultures were grown for 23 hours at 30°C and used to inoculate 35 mL of SC-His
20 media containing 2 % raffinose. The subcultures were grown for 8 hours at 30°C, and their OD_{600s} were determined. For each strain, a volume of cells giving an OD x volume equal to 40 was pelleted, resuspended in 10 mL of SC-His media containing 2%
galactose, and used to inoculate a total volume of 100 mL of this media. Cultures were
grown for 17 hours at 30°C and 250 rpm. Cells were then pelleted, rinsed in 0.85% NaCl,
25 and repelleted. Cell pellets (190 mg) were suspended in 380 μ L of 100 mM potassium phosphate buffer, pH 7.5, and an equal volume (pellet plus buffer) of pre-rinsed glass beads (Sigma, 150-212 microns) was added. This mixture was vortexed for 30 seconds and placed on ice for 1 minute, and the vortexing/cooling cycle was repeated 7 additional
30 times. The cells were then centrifuged for 6 minutes at 5,000 g and the supernatant was removed to a fresh tube. The beads/pellet were washed twice with 300 μ L of buffer and centrifuged, and the supernatants joined with the first supernatant.

An anaerobically-grown culture of *E. coli* strain DH10B was used as a positive control for D-LDH assays. A culture of this strain was grown to saturation overnight and diluted 1:20 the following morning in fresh LB media. The culture was grown anaerobically at 37°C for 7.5 hours. Cells were pelleted, washed with 0.85 % NaCl, and repelleted. Cells were disrupted using BugBuster™ Protein Extraction Reagent and Benzonase® (Novagen) as per manufacturer's instructions with a 20-minute incubation at room temperature. After centrifugation at 16,000g and 4°C, the supernatant was transferred to a new tube and used in the activity assay.

Total protein content of cell extracts was quantified using a microplate Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The D-LDH and D-LDH/PCT constructs in YPH500, the pESC-His negative control in YPH500, and the anaerobically-grown *E. coli* strain were tested for their ability to catalyze the conversion of pyruvate to lactate by assaying the concurrent oxidation of NADH to NAD. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.5, 0.2 mM NADH, and 0.5-1.0 µg of cell extract. The reaction was started by the addition of sodium pyruvate to a final concentration of 5 mM, and the decrease in absorbance at 340 nm was measured over 10 minutes. When 0.5 µg of total cell extract protein of the negative control strain was added to the reaction mixture, a decrease in absorbance from -0.01 to -0.02 was seen over 200 seconds. A decrease in absorbance from -0.21 to -0.47 and -0.20 to -0.47 over 200 seconds was seen, respectively, for cell extract from the YPH500/pESC-His/D-LDH or YPH500/pESC-His/D-LDH/PCT strains. 0.5 µL (7.85 µg) of cell extract from the anaerobically-grown *E. coli* strain showed a decrease in absorbance very similar to that for 1 µg of cell extract of the YPH500/pESC-His/D-LDH/PCT strain. When 4 µg of cell extract was used, the YPH500/pESC-His/D-LDH/PCT strain showed a decrease in absorbance from -0.18 to -0.60 over 10 minutes, whereas the negative control strain showed no decrease in absorbance (-0.08 to -0.08).

D. Demonstration of 3-HP production in *S. cerevisiae*

The pESC-Trp/OS19/El, pESC-Leu/EIIa/EIIB, and pESC-His/D-LDH/PCT constructs were transformed into a single strain of *S. cerevisiae* YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). A negative control

strain was also developed by transformation of the pESC-Trp, pESC-Leu, and pESC-His vectors into a single YPH500 strain. Transformation reactions were plated on SC-Trp-Leu-His media. Individual yeast colonies were screened by colony PCR for the presence or absence of nucleic acid corresponding to each construct.

5 The strain carrying all six genes and the negative control strain were grown in 5 mL of SC-Trp-Leu-His media containing 2% glucose. These cultures were grown for 31 hours at 30°C, and 2 mL was used to inoculate 50 mL of the same media. The subcultures were grown for 19 hours at 30°C, and their OD600s were determined. For each strain, a volume of cells giving an OD x volume equal to 100 was pelleted, washed
10 with SC-Trp-Leu-His media with no carbon source, and repelleted. The cells were suspended in 10 mL of SC-Trp-Leu-His media containing 2% galactose and 2% raffinose and used to inoculate a total volume of 250 mL of this media. The cultures were grown in bottles at 30°C with no shaking, and samples were taken at 0, 4.5, 20, 28.5, 45, and 70
15 hours. Samples were spun down to remove cells and the supernatant was filtered using 0.45 micron Acrodisc Syringe Filters (Pall Gelman Laboratory, Ann Arbor, MI).

100 microliters of the filtered broth was used to derive CoA esters of any lactate or 3-HP in the broth using 6 micrograms of purified propionyl-CoA transferase, 50 mM potassium phosphate buffer (pH 7.0), and 1 mM acetyl-CoA. The reaction was allowed
20 to proceed at room temperature for 15 minutes and was stopped by adding 1 volume 10% trifluoroacetic acid. The reaction mixtures were purified using Sep Pak C18 columns as previously described and analyzed by LC/MS.

Example 14 Constructing a Biosynthetic Pathway that

Produces Organic Acids from β -alanine

25 One possible pathway to 3-HP from β -alanine involves the use of a polypeptide having CoA transferase activity (e.g., an enzyme from a class of enzymes that transfers a CoA group from one metabolite to the other). As shown in Figure 54, β -alanine can be converted to β -alanyl-CoA using a polypeptide having CoA transferase activity and CoA donors such as acetyl-CoA or propionyl-CoA. Alternatively, β -alanyl-CoA can be
30 generated by the action of a polypeptide having CoA synthetase activity. The β -alanyl-CoA can be deaminated to form acrylyl-CoA by a polypeptide having β -alanyl-CoA

ammonia lyase activity. The hydration of acrylyl-CoA at the β position to yield 3-HP-CoA can be carried out by a polypeptide having 3-HP-CoA dehydratase activity. The 3-HP-CoA can act as a CoA donor for β -alanine, a reaction that can be catalyzed a polypeptide having CoA transferase activity, thus yielding 3-HP as a product.

- 5 Alternatively, 3-HP-CoA can be hydrolyzed to yield 3-HP by a polypeptide having specific CoA hydrolase activity.

Methods for isolating, sequencing, expressing, and testing the activity of a polypeptide having CoA transferase activity are described herein.

10 A. Isolation of a polypeptide having β -alanyl-CoA Ammonia Lyase Activity

Polypeptides having β -alanyl-CoA ammonia lyase activity can catalyze the conversion of β -alanyl-CoA into acrylyl-CoA. The activity of such polypeptides has been described by Vagelos *et al.* (*J. Biol. Chem.*, 234:490-497 (1959)) in *Clostridium propionicum*. This polypeptide can be used as part of the acrylate pathway in *Clostridium*
15 *propionicum* to produce propionic acid.

- C. propionicum* was grown at 37°C in an anoxic medium containing 0.2% yeast extract, 0.2% trypticase peptone, 0.05% cysteine, 0.5% β -alanine, 0.4% VRB-salts, 5 mM potassium phosphate, pH 7.0. The cells were harvested after 12 hours and washed twice with 50 mM potassium phosphate (Kpi), pH 7.0. About 2 g of wet packed cells were re-suspended in 40 mL of Kpi, pH 7.0, 1mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT (Buffer
20 A), and homogenized by sonication at about 85-100 W power using a 3mm tip (Branson sonifier 250). Cell debris was removed by centrifugation at 100,000g for 45 minutes in a Centricon T-1080 Ultra centrifuge, and the cell free extract (~ 110 U/mg activity) was subjected to anion exchange chromatography on Source-15Q-material. The Source-15Q
25 column was loaded with 32 mL of cell free extract. The column was developed by a linear gradient of 0 M to 0.5 M NaCl within 10 column volumes. The polypeptide eluted between 70-110 mM NaCl.

- The solution was adjusted to a final concentration of 1 M $(NH_4)_2SO_4$ and applied onto a Resource-Phe column-equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. The
30 polypeptide did not bind to this column.

The final preparation was obtained after concentration in an Amicon chamber (filter cut-off 30 kDa). The functional polypeptide is composed of four polypeptide subunits, each having a molecular mass of 16 kDa. The polypeptide had a final specific activity of 1033 U/mg in the standard assay (see below).

5 The polypeptide sample after every purification step was separated on a 15% SDS-PAGE gel. The gel was stained with 0.1% Coomassie R 250, and the destaining was achieved by using 7.1% acetic acid/5% ethanol solution.

The polypeptide was desalted by RP-HPLC and subjected to N-terminal sequencing by gas phase Edman degradation. The results of this analysis yielded a 35
10 amino acid N-terminal sequence of the polypeptide. The sequence was as follows: MV-GKKVVHHLMMSAKDAHYTGNLVNGARIVNQWGD (SEQ ID NO:177).

B. Amplification of a Gene Fragment

The 35 amino acid sequence of the polypeptide having β -alanine-CoA ammonia
15 lyase activity was used to design primers with which to amplify the corresponding DNA from genome of *C. propionicum*. Genomic DNA from *C. propionicum* was isolated using the Gentra Genomic DNA isolation Kit (Gentra Systems, Minneapolis) following the genomic DNA protocol for gram-positive bacteria. A codon usage table for *Clostridium propionicum* was used to back translate the seven amino acids on either end
20 of the amino acid sequence to obtain 20-nucleotide degenerate primers:

ACLF: 5'-ATGGTWGGYAARAARGTWGT -3' (SEQ ID NO:178)

ACLR: 5'-TCRCCCCAYTGRTTWACRAT -3' (SEQ ID NO:179)

The primers were used in a 50 μ L PCR reaction containing 1X Taq PCR buffer, 0.6 μ M each primer, 0.2 mM each dNTP, 2 units of Taq DNA polymerase (Roche
25 Molecular Biochemicals, Indianapolis, IN), 2.5% (v/v) DMSO, and 100 ng of genomic DNA. PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 58°C, 4 cycles at 56°C, 4 cycles at 54°C, and 24 cycles at 52°C. Each cycle used an initial 30 second denaturing step at 94°C and a 1.25 minute extension at 72°C, and the program had an initial denaturation step at 94°C for 2 minutes and final
30 extension at 72°C for 5 minutes. The amounts of PCR primer used in the reaction were increased three-fold above typical PCR amounts due to the amount of degeneracy in the

3' end of the primer. In addition, separate PCR reactions containing each individual primer were made to identify PCR product resulting from single degenerate primers. Twenty μ L of each PCR product was separated on a 2.0% TAE (Tris-acetate-EDTA)-agarose gel.

5 A band of about 100 bp was produced by the reaction containing both the forward and reverse primers, but was not present in the individual forward and reverse primer control reactions. This fragment was excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Four microliters of the purified band was ligated into pCRII-TOPO vector and transformed by a heat-shock method into TOP10 *E. coli*
10 cells using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 50 μ g/mL of kanamycin and 50 μ g/mL of 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside (X-gal). Individual, white colonies were resuspended in 25 μ L of 10 mM Tris and heated for 10 minutes at 95°C to break open the bacterial cells. Two microliters of the heated cells were used in a 25 μ L PCR reaction
15 using M13R and M13F universal primers homologous to the pCRII-TOPO vector. The PCR mix contained the following: 1X Taq PCR buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR reaction was performed in a MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 52°C for 1 minute,
20 and 72°C for 1.25 minutes; and a final extension for 7 minutes at 72°C. Plasmid DNA was obtained (QIAprep Spin Miniprep Kit, Qiagen) from cultures of colonies showing the desired insert and was used for DNA sequencing with M13R universal primer. The following nucleic acid sequence was internal to the degenerate primers and corresponds to a portion of the 35 amino acid residue sequence: 5'-ACATCATTTAATGATGA-
25 GCGCAAAAGATGCTCACTATACTGGAACTTAGTAAACGGCGCTAGA-3' (SEQ ID NO:180).

C. Genome Walking to Obtain the Complete Coding Sequence

30 Primers for conducting genome walking in both upstream and downstream directions were designed using the portion of the nucleic acid sequence that was internal to the degenerate primers. The primer sequences were as follows:

ACLGSP1F: 5'-GTACATCATTTAATGATGAGCGCAAAAGATG-3' (SEQ ID NO:181)

ACLGSP2F: 5'-GATGCTCACTATACTGGAACTTAGTAAAC-3' (SEQ ID NO:182)

5 ACLGSP1R: 5'-ATTCTAGCGCCGTTTACTAAGTTTCCAG-3' (SEQ ID NO:183)

ACLGSP2R: 5'-CCAGTATAGTGAGCATCTTTGCGCTCATC-3' (SEQ ID NO:184)

GSP1F and GSP2F are primers facing downstream, GSP1R and GSP2R are primers facing upstream, and GSP2F and GSP2R are primers nested inside GSP1F and GSP1R, respectively. Genome walking libraries were constructed according to the manual for CLONTECH's Universal Genome Walking Kit (CLONTECH Laboratories, Palo Alto, CA), with the exception that the restriction enzymes *Ssp* I and *Hinc* II were used in addition to *Dra* I, *EcoR* V, and *Pvu* II. PCR was conducted in a Perkin Elmer 9700 Thermocycler using the following reaction mix: 1X XL Buffer II, 0.2 mM each dNTP, 1.25 mM Mg(OAc)₂, 0.2 μM each primer, 2 units of rTth DNA polymerase XL (Applied Biosystems, Foster City, CA), and 1 μL of library per 50 μL reaction. First round PCR used an initial denaturation at 94°C for 5 seconds; 7 cycles consisting of 2 sec at 94°C and 3 min at 70°C; 32 cycles consisting of 2 sec at 94°C and 3 min at 64°C; and a final extension at 64°C for 4 min. Second round PCR used an initial denaturation at 94°C for 15 seconds; 5 cycles consisting of 5 sec at 94°C and 3 min at 70°C; 26 cycles consisting of 5 sec at 94°C and 3 min at 64°C; and a final extension at 66°C for 7 min. Twenty μL of each first and second round product was run on a 1.0% TAE-agarose gel. In the second round PCR for the forward reactions, a 1.4 Kb band was obtained for *Dra* I, a 1.5 Kb band for *Hinc* II, a 4.0 Kb band for *Pvu* II, and 2.0 and 2.6 Kb bands were obtained for *Ssp* I. In the second round PCR for the reverse reactions, a 1.5 Kb band was obtained for *Dra* I, a 0.8 Kb band for *EcoR* V, a 2.0 Kb band for *Hinc* II, a 2.9 Kb band for *Pvu* II, and a 1.5 Kb band was obtained for *Ssp* I. Several of these fragments were gel purified, cloned, and sequenced.

The coding sequence of the polypeptide having β-alanyl-CoA ammonia lyase activity is set forth in SEQ ID NO:162. This coding sequence encodes the amino acid sequence set forth in SEQ ID NO:160. The coding sequence was cloned and expressed in

bacterial cells. A polypeptide with the expected size was isolated and tested for enzymatic activity.

The isolation of a nucleic acid molecule encoding a polypeptide having 3-HP-CoA dehydratase activity (e.g., the seventh enzymatic activity in Figure 54, which can be accomplished with a polypeptide having the amino acid sequence set forth in SEQ ID NO:41) is described herein. This polypeptide in combination with a polypeptide having CoA transferase activity (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO:2) and a polypeptide having β -alanyl-CoA ammonia lyase activity (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO: 160) provides one method of making 3-HP from β -alanine.

**Example 15 Constructing a Biosynthetic Pathway that
Produces Organic Acids from β -alanine**

In another pathway, β -alanine generated from aspartate can be deaminated by a polypeptide having 4, 4-aminobutyrate aminotransferase activity (Figure 55). This reaction also can regenerate glutamate that is consumed in the formation of aspartate. The deamination of β -alanine can yield malonate semialdehyde, which can be further reduced to 3-HP by a polypeptide having 3-hydroxypropionate dehydrogenase activity or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity. Such polypeptides can be obtained as follows.

A. Cloning gabT (4-aminobutyrate aminotransferase) from *C. acetobutylicum*

The following PCR primers were designed based on a published sequence for a gabT gene from *Clostridium acetobutylicum* (GenBank# AE007654):

Cac aba nco sen: 5'-GAGCCATGGAAGAAATAAATGCTAAAG- 3' (SEQ ID NO:185)

Cac aba bam anti: 5'-AGAGGATGGCTTTTTTAAATCGCTATTC- 3' (SEQ ID NO:186)

The primers introduced a *Nco*I site at the 5' end and a *Bam*HI site at the 3' end. A PCR reaction was set up using chromosomal DNA from *C. acetobutylicum* as the template.

	H ₂ O	80.75 μ L	PCR Program
	Taq Plus Long 10x Buffer	10 μ L	94° C 5 minutes
	dNTP mix (10 mM)	3 μ L	25 cycles of:
	Cac aba nco sen (20 mM)	2 μ L	94° C 30 seconds
5	Cac aba bam anti (20 mM)	2 μ L	50° C 30 seconds
	<i>C. acetobutylicum</i> DNA (~100 ng)	1 μ L	72° C 80 seconds + 2
	Taq Plus Long (5 U/mL)	1 μ L	seconds/cycle
	Pfu (2.5 U/mL)	0.25 μ L	1 cycle of:
			68° C 7 minutes
10			4° C until use

Upon agarose gel analysis a single band was observed of ~1.3 Kb in size. This fragment was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into pCRII TOPO using the TOPO Zero Blunt PCR cloning kit (Invitrogen, Carlsbad, CA). 1 μ L of the pCRII TOPO ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 μ g/mL) plates. Single colonies of the transformant grown overnight in LB/kanamycin media, and the plasmid DNA was extracted using a Mini prep kit (Qiagen, Valencia, CA). The super-coiled plasmid DNA was separated on a 1% agarose gel digested, and the colonies with insert were selected. The insert was sequenced to confirm the sequence and its quality.

The plasmid having the correct insert was digested with restriction enzyme *Nco* I and *Bam*HI. The digested insert was gel isolated and ligated to pET28b expression vector that was also restricted with *Nco* I and *Bam*HI enzymes. 1 μ L of ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were recovered for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 μ g/mL) plates. The super-coiled plasmid DNA was separated on a 1% agarose gel digested, and the colonies with insert were selected. The plasmid with the insert was isolated using a Mini prep kit (Qiagen, Valencia, CA), and 1 μ L of this plasmid DNA was used to transform electrocompetent BL21(DE3) (Novagen, Madison, WI). These cells were used to check the expression of a polypeptide having 4-aminobutyrate aminotransferase activity.

B. Cloning mmsB (3-hydroxyisobutyrate dehydrogenase) from *P. aeruginosa*

The following PCR primers was designed based on a published sequence for a mmsB gene from *Pseudomona aeruginosa* (GenBank# M84911):

Ppu hid nde sen: 5'-ATACATATGACCGACCGACATCGCATT-3' (SEQ ID NO:186)

5 Ppu hid sal anti: 5'-ATAGTCGACGGGTCAGTCCTTGCCGCG-3' (SEQ ID NO:187)

The primers introduced a *Nde* I site at the 5' end and a *Bam*HI site at the 3' end.

H ₂ O	80.75 µL	PCR Program
Taq Plus Long 10x Buffer	10 µL	94° C 5 minutes
dNTP mix (10 mM)	3 µL	25 cycles of: 94° C 30 seconds 55° C 30 seconds 72° C 90 seconds + 2 seconds/cycle
Ppu hid.nde sen (20 µM)	2 µL	68° C 7 minutes
Ppu hid sal anti (20 µM)	2 µL	4° C until use
<i>C. acetobutylicum</i> DNA (~100 ng)	1 µl	
Taq Plus Long (Stratagene, La Jolla, CA)	1 µL	
Pfu (Stratagene, La Jolla, CA)	0.25 µL	

10 A PCR reaction was set up using chromosomal DNA from *P. aeruginosa* as the template. Chromosomal DNA was obtained from ATCC (Manassas, VA) *P. aeruginosa* 17933D.

15 Upon agarose gel analysis, a single band was observed of ~1.6 Kb in size. This fragment was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into pCRII TOPO using the TOPO Zero Blunt PCR cloning kit (Invitrogen, Carlsbad, CA). 1 µL of the pCRII TOPO ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were recovered for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 µg/mL) plates. Single colonies of the transformant grown overnight in LB/kanamycin media, and the plasmid DNA was extracted using a Mini prep kit (Qiagen, Valencia, CA). The super-coiled plasmid DNA

was separated on a 1% agarose gel and digested, and the colonies with insert were selected. The insert was sequenced to confirm the sequence and its quality.

The plasmid having the correct insert was digested with restriction enzyme *Nde* I and *Bam*HI. The digested insert was gel isolated and ligated to pET30a expression vector that was also restricted with *Nde* I and *Bam*HI enzymes. 1 µL of ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were recovered for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 µg/mL) plates. The super-coiled plasmid DNA was separated on a 1% agarose gel and digested, and the colonies with insert were selected. The plasmid with the insert was isolated using a Mini prep kit (Qiagen, Valencia, CA), and 1 µl of this plasmid DNA was used to transform electrocompetent BL21(DE3) (Novagen, Madison, WI). These cells were used to check the expression of a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

15

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

20

WHAT IS CLAIMED IS:

1. A cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.
- 5 2. The cell of claim 1, wherein said cell comprises an activity selected from the group consisting of E1 activator activity, E2 α activity, and E2 β activity.
3. The cell of claim 1, wherein said cell comprises 3-hydroxypropionyl-CoA
10 dehydratase activity.
4. The cell of claim 1, wherein said cell comprises CoA transferase activity.
5. The cell of claim 1, wherein said cell comprises an exogenous nucleic acid
15 comprising:
 - (a) a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; or
 - (b) a nucleic acid sequence that shares at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162,
20 or 163.
6. The cell of claim 1, wherein said cell comprises 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
- 25 7. The cell of claim 1, wherein said cell comprises lipase activity.
8. The cell of claim 1, wherein said cell produces 3-HP.
9. The cell of claim 1, wherein said cell produces an ester of 3-HP.
- 30

10. The cell of claim 9, wherein said ester is selected from the group consisting of methyl 3-hydroxypropionate, ethyl 3-hydroxypropionate, propyl 3-hydroxypropionate, butyl 3-hydroxypropionate, and 2-ethylhexyl 3-hydroxypropionate.
- 5 11. The cell of claim 1, wherein said cell comprises CoA synthetase activity.
12. The cell of claim 1, wherein said cell comprises poly hydroxyacid synthase activity.
- 10 13. The cell of claim 1, wherein said cell produces polymerized 3-HP.
14. The cell of claim 1, wherein said cell is prokaryotic.
15. The cell of claim 1, wherein said cell is selected from the group consisting of
15 yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
16. A cell comprising CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity.
- 20 17. The cell of claim 16, wherein said cell comprises an activity selected from the group consisting of E1 activator activity, E2 α activity, and E2 β activity.
18. The cell of claim 16, wherein the cell produces polymerized acrylate.
- 25 19. The cell of claim 16, wherein said cell is prokaryotic.
20. The cell of claim 16, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
- 30 21. A cell comprising CoA transferase activity, lactyl-CoA dehydratase activity, and lipase activity.

22. The cell of claim 21, wherein said cell comprises an activity selected from the group consisting of E1 activator activity, E2 α activity, and E2 β activity.
- 5 23. The cell of claim 21, wherein said cell produces an ester of acrylate.
24. The cell of claim 23, wherein said ester is selected from the group consisting of methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate.
- 10 25. The cell of claim 21, wherein said cell is prokaryotic.
26. The cell of claim 21, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
- 15 27. An polypeptide comprising an amino acid sequence selected from the group consisting of:
- (a) a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161;
 - (b) a sequence having at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161;
 - 20 (c) a sequence that has at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161;
 - (d) a sequence that has at least 65 percent sequence identity with at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 25 37, 39, 41, 141, 160, or 161; and
 - (e) a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 that contains at least one conservative substitution.
28. A nucleic acid molecule comprising a nucleic acid sequence that encodes the
- 30 polypeptide of claim 27.

29. A transformed cell comprising at least one exogenous nucleic acid molecule, wherein said molecule comprises a nucleic acid sequence that encodes the polypeptide of claim 27.
- 5 30. The cell of claim 29, wherein the cell produces 3-HP.
31. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes an E2 α polypeptide of an enzyme having lactyl-CoA dehydratase activity.
- 10 32. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes an E2 β polypeptide of an enzyme having said lactyl-CoA dehydratase activity.
33. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity or CoA transferase
15 activity.
34. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
- 20 35. The cell of claim 29, wherein the cell comprises lipase activity.
36. The cell of claim 29, wherein the cell produces an ester of 3-HP.
- 25 37. The cell of claim 36, wherein said ester is selected from the group consisting of methyl 3-hydroxypropionate, ethyl 3-hydroxypropionate, propyl 3-hydroxypropionate, butyl 3-hydroxypropionate, and 2-ethylhexyl 3-hydroxypropionate.
38. The cell of claim 29, wherein said cell comprises CoA synthetase activity.
- 30 39. The cell of claim 29, wherein said cell produces polymerized 3-HP.

40. The cell of claim 29, wherein said cell is prokaryotic.
41. The cell of claim 29, wherein said cell is selected from the group consisting of
5 *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
42. The cell of claim 29, wherein the cell is a yeast cell.
43. A specific binding agent that specifically binds to the polypeptide of claim 27.
- 10 44. An isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of: —————
- (a) a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163;
- 15 (b) a sequence having at least 10 contiguous nucleotides of a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163;
- (c) a sequence that has at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163;
- (d) a sequence that has at least 65 percent sequence identity with at least 10
20 contiguous nucleotides of a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; and
- (e) a sequence that hybridize under moderately stringent conditions a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163.
- 25 45. A production cell comprising an isolated nucleic acid molecule of claim 44 that is exogenous to said production cell.
46. The cell of claim 45, wherein said isolated nucleic acid molecule encodes a polypeptide having an enzymatic activity selected from the group consisting of CoA
30 transferase activity, lactyl-CoA dehydratase activity, CoA synthase activity, CoA

dehydratase activity, dehydrogenase activity, malonyl-CoA reductase activity, and 3-hydroxypropionyl-CoA dehydratase activity.

47. A method of producing a polypeptide, comprising culturing the cell of claim 45
5 under conditions that allow said cell to produce said polypeptide, wherein said polypeptide is produced.

48. A method for making 3-HP, said method comprising culturing at least one cell
comprising at least one exogenous nucleic acid molecule that encodes at least one
10 polypeptide that is capable of producing said 3-HP from PEP under conditions such that said 3-HP is produced.

49. The method of claim 48, wherein said cell is selected from the group consisting of
yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

15 50. The method of claim 48, wherein 3-HP is made by a biosynthetic route that utilizes a β -alanine intermediate.

51. The method of claim 48, wherein 3-HP is made by a biosynthetic route that
20 utilizes a malonyl-CoA intermediate.

52. The method of claim 48, wherein 3-HP is made by a biosynthetic route that utilizes a lactate intermediate.

25 53. A method for making 3-HP, said method comprising culturing at least one cell comprising at least one exogenous nucleic acid molecule that encodes at least one polypeptide that is capable of producing said 3-HP from lactate under conditions such that said 3-HP is produced.

30 54. The method of claim 53, wherein said cells are selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

55. A method for making 3-HP, said method comprising culturing at least one cell under conditions wherein said cell produces said 3-HP, said cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.
56. The method of claim 55, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
57. The method of claim 55, wherein said cell comprises CoA transferase activity.
58. The method of claim 55, wherein said cell comprises 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
59. A method for making 3-HP, said method comprising:
- a) contacting lactate with a first polypeptide having CoA transferase activity to form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA,
 - c) contacting said acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-HP-CoA, and
 - d) contacting said 3-HP-CoA with said first polypeptide to form said 3-HP or with a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form said 3-HP.
60. A method for making polymerized 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said polymerized 3-HP, said cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.
61. The method of claim 60, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

62. The method of claim 60, wherein said cell comprises CoA synthetase activity.
63. The method of claim 60, wherein said cell comprises poly hydroxyacid synthase activity.
- 5 64. A method for making polymerized 3-HP, said method comprising:
- a) contacting lactate with a first polypeptide having CoA synthetase activity to form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA
 - 10 dehydratase activity to form acrylyl-CoA,
 - c) contacting said acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA, and
 - d) contacting said 3-hydroxypropionic acid-CoA with a fourth polypeptide having
 - 15 poly hydroxyacid synthase activity to form said polymerized 3-HP.
65. A method for making an ester of 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said ester, said cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.
- 20 66. The method of claim 65, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
67. The method of claim 65, wherein said cell comprises CoA transferase activity.
- 25 68. The method of claim 65, wherein said cell comprises 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
69. A method for making an ester of 3-HP, said method comprising:
- a) contacting lactate with a first polypeptide having CoA transferase activity to
 - 30 form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA

dehydratase activity to form acrylyl-CoA,

c) contacting said acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA,

d) contacting said 3-hydroxypropionic acid-CoA with said first polypeptide to
5 form 3-HP or a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form 3-HP, and

e) contacting said 3-HP with a fifth polypeptide having lipase activity to form said ester.

10 70. A method for making polymerized acrylate, said method comprising culturing a cell under conditions wherein said cell produces said polymerized acrylate, said cell comprising CoA synthetase activity and lactyl-CoA dehydratase activity.

71. The method of claim 70, wherein said cell is selected from the group consisting of
15 yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

72. The method of claim 70, wherein said cell comprises poly hydroxyacid synthase activity.

20 73. A method for making polymerized acrylate, said method comprising:

a) contacting lactate with a first polypeptide having CoA synthetase activity to form lactyl-CoA,

b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, and

25 c) contacting said acrylyl-CoA with a third polypeptide having poly hydroxyacid synthase activity to form said polymerized acrylate.

74. A method for making an ester of acrylate, said method comprising culturing a cell under conditions wherein said cell produces said ester, said cell comprising CoA
30 transferase activity and lactyl-CoA dehydratase activity.

75. The method of claim 74, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
76. The method of claim 74, wherein said cell comprises lipase activity.
- 5 77. A method for making an ester of acrylate, said method comprising:
- a) contacting lactate with a first polypeptide having CoA transferase activity to form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA
 - 10 dehydratase activity to form acrylyl-CoA,
 - c) contacting said acrylyl-CoA with said first polypeptide to form acrylate, and
 - d) contacting said acrylate with a third polypeptide having lipase activity to form said ester.
-
- 15 78. A method for making 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said 3-HP, said cell comprising at least one exogenous nucleic acid that encodes at least one polypeptide such that said 3-HP is produced from acetyl-CoA and under conditions such that said 3-HP is produced.
- 20 79. The method of claim 78, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
80. A method for making 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said 3-HP, said cell comprising at least one
- 25 exogenous nucleic acid that encodes at least one polypeptide such that said 3-HP is produced from malonyl-CoA and under conditions such that said 3-HP is produced.
81. The method of claim 80, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
- 30 82. A method for making 3-HP, said method comprising culturing a cell under

conditions wherein said cell produces said 3-HP, said cell comprising at least one exogenous nucleic acid that encodes at least one polypeptide such that said 3-HP is produced from β -alanine and under conditions such that said 3-HP is produced.

5 83. The method of claim 82, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

84. A method for making 3-HP, said method comprising culturing cells comprising an exogenous nucleic acid that encodes polypeptides that are capable of producing 3-HP
10 from acetyl-CoA under conditions such that said 3-HP is produced.

85. The method of claim 84, wherein said cells are selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

15 86. A method for making 3-HP, said method comprising culturing cells comprising at least one exogenous nucleic acid that encodes polypeptides that are capable of producing said 3-HP from malonyl-CoA, and under conditions such that said 3-HP is produced.

87. The method of claim 86, wherein said cells are selected from the group consisting
20 of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

88. A method for making 3-HP, said method comprising:
a) contacting acetyl-CoA with a first polypeptide having acetyl-CoA carboxylase activity to form malonyl-CoA, and
25 b) contacting said malonyl-CoA with a second polypeptide having malonyl-CoA reductase activity to form said 3-HP.

89. A method for making 3-HP, said method comprising contacting malonyl-CoA with a polypeptide having malonyl-CoA reductase activity to form said 3-HP.
30

90. A method for making 3-HP, said method comprising:

- a) contacting β -alanine CoA with a first polypeptide having β -alanyl-CoA ammonia lyase activity to form acrylyl-CoA;
- b) contacting said acrylyl-CoA with a second polypeptide having 3HP-CoA dehydratase activity to form said 3-HP-CoA; and
- 5 c) contacting 3-HP-CoA with a third polypeptide having glutamate dehydrogenase to make 3-HP.

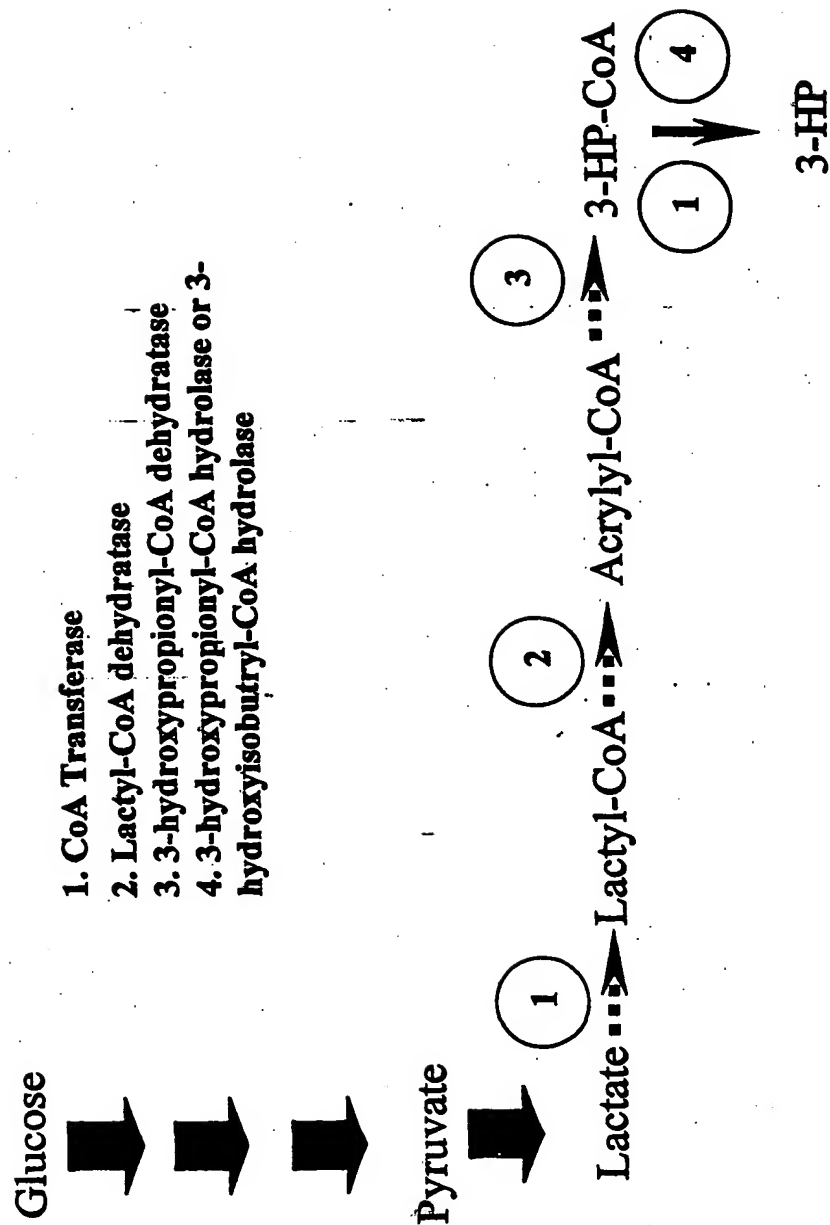
Figure 1

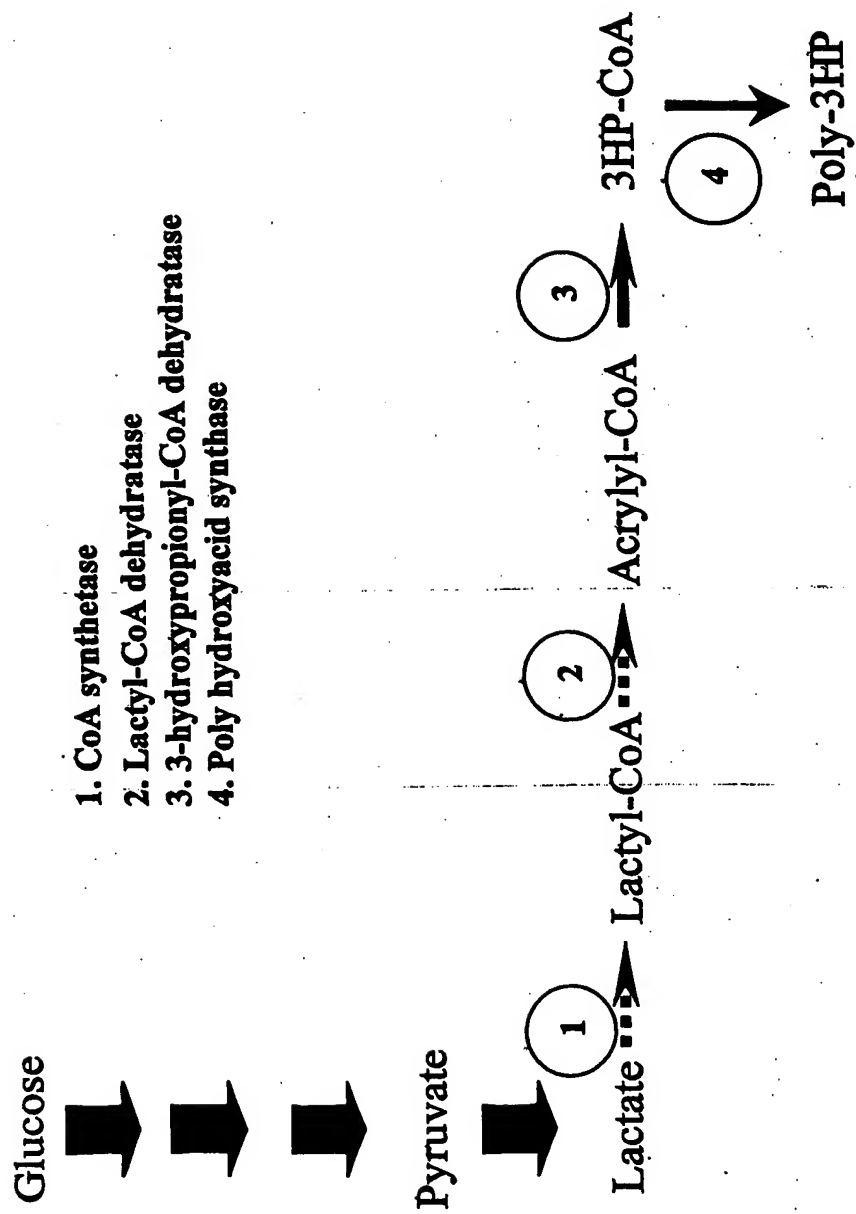
Figure 2

Figure 3

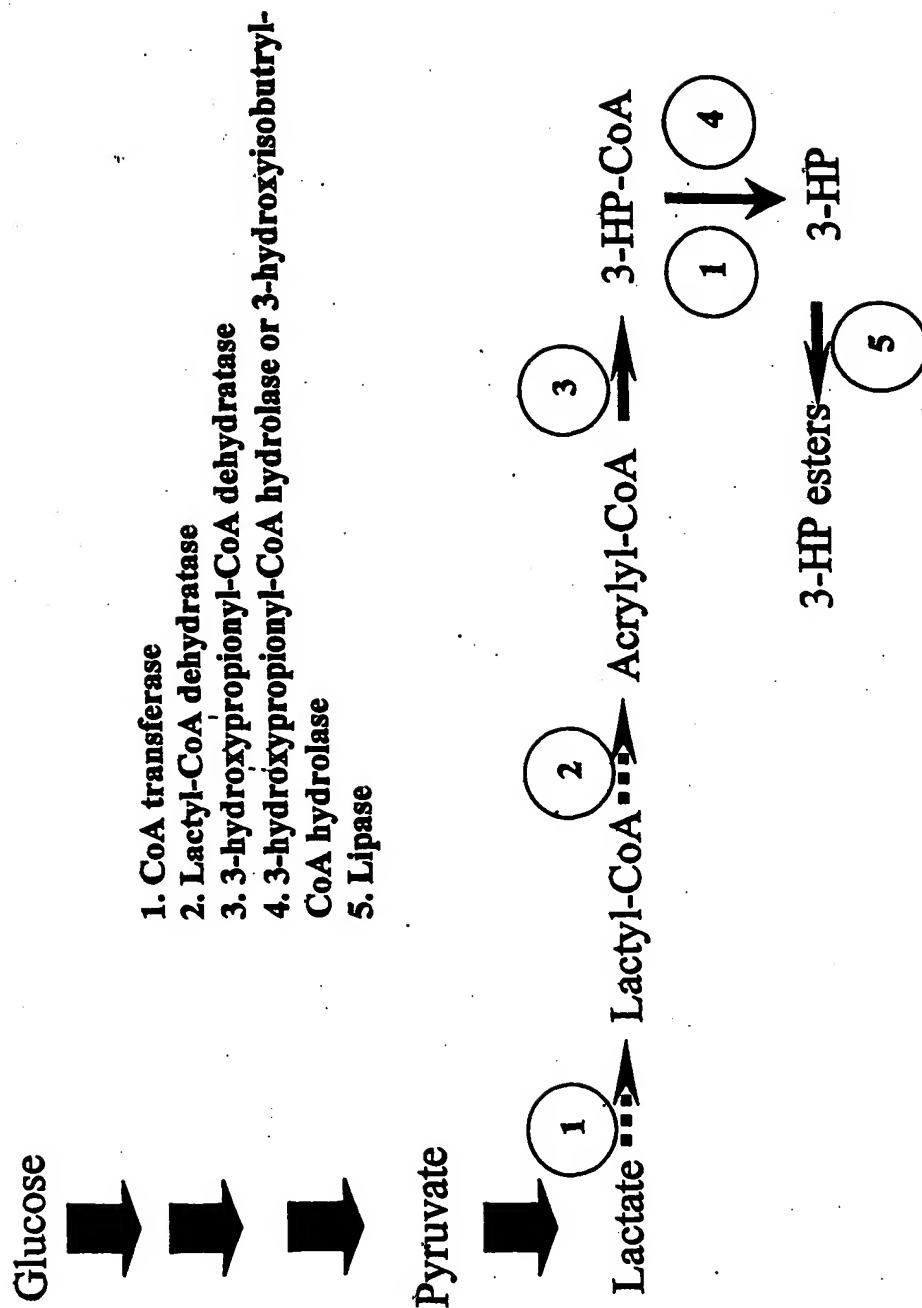


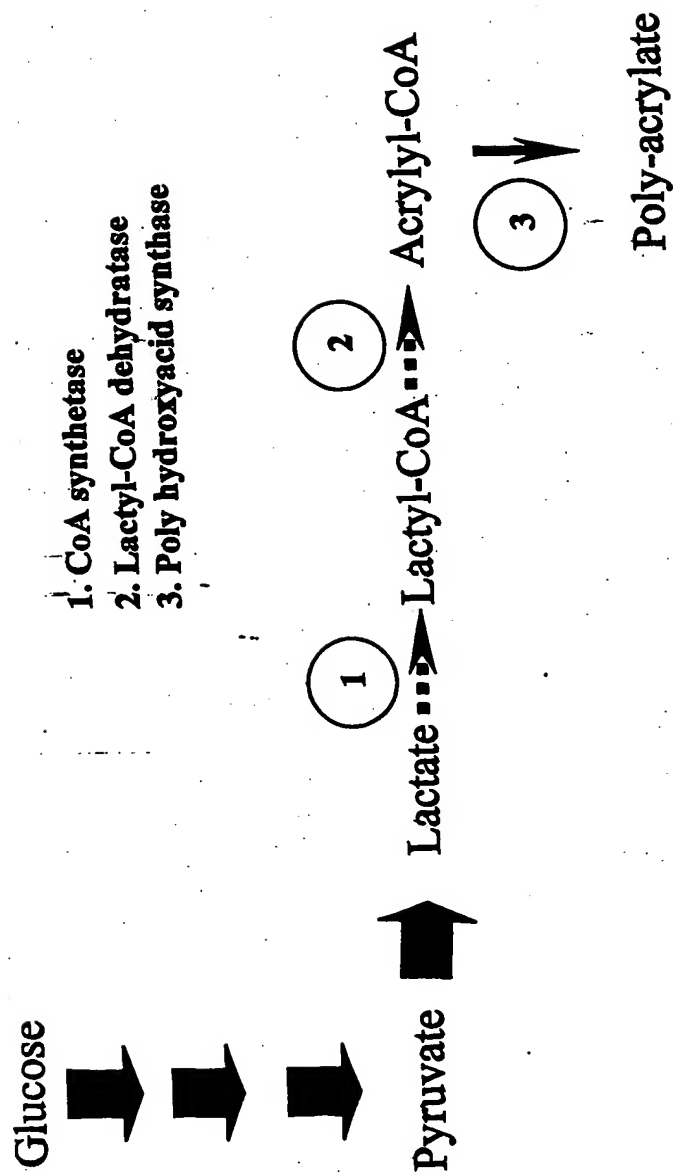
Figure 4

Figure 5

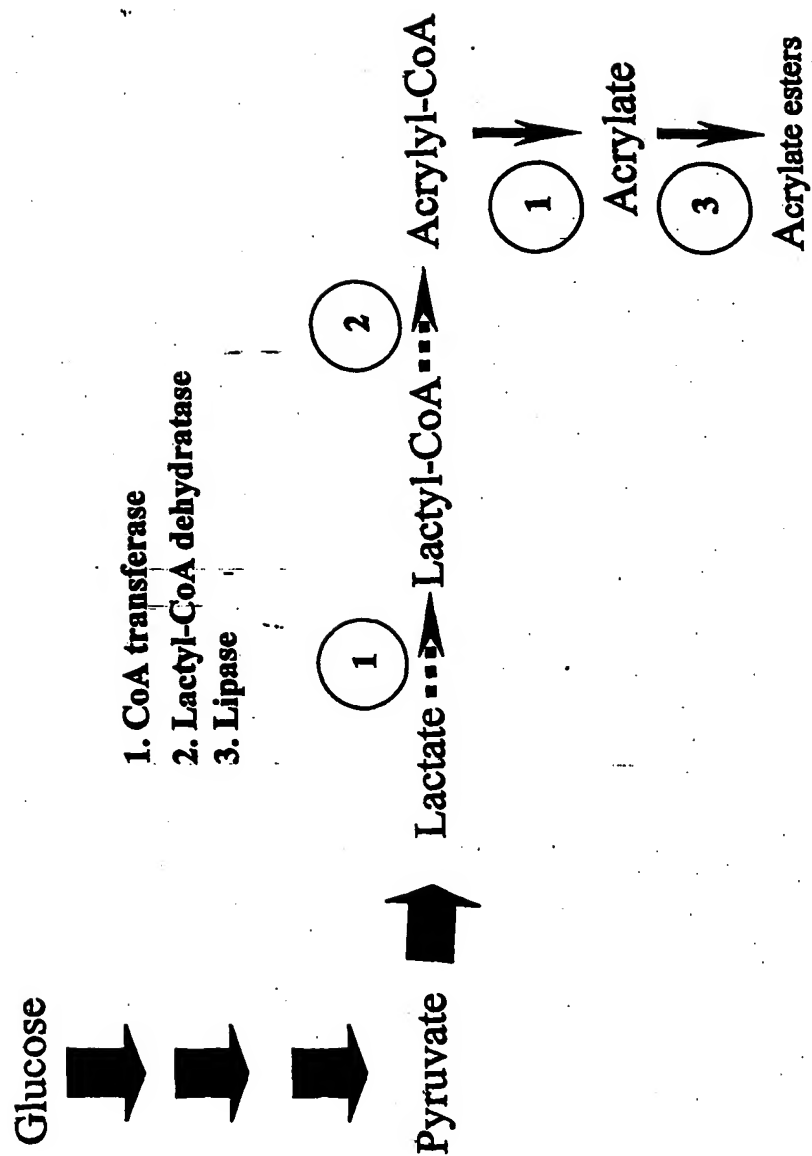


Figure 6

ATGAGAAAAGTAGAAATCATTACAGCTGAACAAGCAGCTCAGCTCGTAAAAGACAACGAC
ACGATTACGTCTATCGGCTTTGTGTCAGCAGCGCCCATCCGGAAGCACTGACCAAAGCTTTG
GAAAAACGGTTCCTGGACACGAACACCCCGCAGAACTTGACCTACATCTATGCAGGCTCT
CAGGGCAAACGCGATGGCCGTGCCGTGAACATCTGGCACACACAGGCCTTTTGAAACGC
GCCATCATCGGTCACTGGCAGACTGTACCGGCTATCGGTAAACTGGCTGTGCGAAAACAAG
ATTGAAGCTTACAACCTTCTCGCAGGGCACGTTGGTCCACTGGTTCCGGCCTTGGCAGGT
CATAAGCTCGGCGTCTTCACCGACATCGGTCTGGAACTTTCTCGATCCCGCTCAGCTC
GGCGGCAAGCTCAATGACGTAACCAAAGAAGACCTCGTCAAACCTGATCGAAGTCGATGGT
CATGAACAGCTTTTCTACCCGACCTTCCCGGTCAAGGTAGCTTTCCTCCGCGGTACGTAT
GCTGATGAATCCGGCAATATCACCATGGACGAAGAAATCGGGCCCTTCGAAAGCACTTCC
GTAGCCCAGGCGGTTCACTGTGGCGGTAAAGTCGTGCTCCAGGTCAAAGAGGTCGTC
GCTCACGGCAGCCTCGACCCGCGCATGGTCAAGATCCCTGGCATCTATGTGCGACTACGTC
GTCGTAGCAGCTCCGGAAGACCATCAGCAGACGTATGACTGCCAATACGATCCGTCCCTC
AGCGGTGAACATCGTGCTCCTGAAGGCGCTACCGATGCAGCTCTCCCATGAGCGCTAAG
AAAATCATCGGCGCGCGCGGCTTTGGAATTGACTGAAAACGCTGTGTCGAACCTCGGC
GTCGGTGCTCCGGAATACGTTGCTTCTGTTGCCGGTGAAGAAGGTATCGCCGATACCATT
ACCTTGACCGTCGAAGGTGGCGCCATCGGTGGCGTACCGCAGGGCGGTGCCCGCTTCGGT
TCGTCCCGCAATGCCGATGCCATCATCGACACACCTATCAGTTCGACTTCTACGATGGC
GGCGGTCTGGACATCGCTTACCTCGGCCTGGCCAGTGCGATGGCTCGGGCAACATCAAC
GTCAGCAAGTTCGGTACTAACGTTGCCGGCTGCGGCGGTTTCCCAACATTTCCAGCAG
ACACCGAATGTTTACTTCTGCGGCACCTTCACGGCTGGCGGCTTGAAAATCGCTGTCGAA
GACGGCAAAGTCAAGATCCTCCAGGAAGGCAAAGCCAAGAAGTTCATCAAAGCTGTGCGAC
CAGATCACTTTCAACGGTTCTATGCAGCCCGCAACGGCAAACACGTTCTCTACATCACA
GAACGCTGCGTATTTGAACTGACCAAAGAAGGCTTGAAACTCATCGAAGTGGCACCAGGC
ATCGATATTGAAAAAGATATCCTCGCTCACATGGACTTCAAGCCGATCATTGATAATCCG
AAACTCATGGATGCCCGCCTCTTCCAGGACGGTGCCATGGGACTGAAAAAATAA (SEQ
ID NO:1)

Figure 7

MRKVEIITAEQAAQLVKDNDTITSIGFVSSAHPEALTKALEKRFLDTNTPQNLTYIYAGS
QGKRDGRAAEHLAHTGLLKRAIIGHWQTVPAIGKLAVENKIEAYNFSQGLVHWFRALAG
HKLGVFTDIGLETFLDPRQLGGKLNDVTKEDLVKLEVDGHEQLFYPTFPVNVAFRLGTY
ADESGNITMDEEIGPFESTSVAQAVHNCGGKVVVQKDVVAHGSLDPRMVKIPGIYVDYV
VVAAPEDHQOTYDCEYDPSLSGEHRAPEGATDAALPMSAKKIIIGRRGALELTENAVVNLG
VGAPEYVASVAGEEGIADTITLTVEGGAIGGVPOGGARFGSSRNADAIIDHTYQFDFYDG
GGLDIAYLGLAQCDSGNINVSKEFTNVAGCGGFPNISQQTPNVYFCGTFTAGGLKIAVE
DGKVKILOEGKAKKFIKAVDQITFNGSYAARNGKHVLYITERCVFELTKEGLKLEIVAPG
IDIEKDILAHMDFKPIIDNPKLMDARLEQDGPMLKK (SEQ ID NO:2)

Figure 8

SEQ ID NO:1	1 atgagaaaagtagaaatcattacagctgaacaagcagctc--agctcgta
SEQ ID NO:3	1 -----gtgccggtcctgtcgccacaggaagcggtaga--attatatt
SEQ ID NO:4	1 atgccgattctctcaaaaaatatgggcggctccagcagctggaatcttgag
SEQ ID NO:5	1 -----atgaa-----tgca
SEQ ID NO:1	49 aaagacaacgacacgattacgtctatcggtttgtcagcagcgcccatcc
SEQ ID NO:3	40 cccgacgaagcaacactttgtgtgttaggcgctg---gcggcggtattct
SEQ ID NO:4	51 aaaaactccgagaaatgctcatcaaatgaggctaattctcaatga-catcc
SEQ ID NO:5	10 aaaga-----atta-----atcg-----
SEQ ID NO:1	99 ggaagcactgaccaaaagctttggaaaaacggttctctg-----
SEQ ID NO:3	87 ggaag-----ccaccacggt--aattactgctcttgctgataaatataa
SEQ ID NO:4	100 tcgatgaaagcaaaagctttt-----aactctgc-----
SEQ ID NO:5	23 -----
SEQ ID NO:1	136 ---gacacgaacaccccgagaaacttgacctacatctatgcag-gctctc
SEQ ID NO:3	129 acagactcaaacaccacgt--aatttatcgattattagtcctaa-cagggc
SEQ ID NO:4	129 -----cgaagaagccgtgaaggatattccagat-aatgcaaaagctttt
SEQ ID NO:5	23 -----ctcgccgaatt-----
SEQ ID NO:1	182 agggcāaacgcgatggcggctgacatctggcacacacaggcctt
SEQ ID NO:3	176 ttggcgatcgccgcgaccggtggtattagtcctctggcgcaagaaggctg
SEQ ID NO:4	171 a-----gttggc--ggcttcggactatgcgg-aatcccagaaaaat
SEQ ID NO:5	34 -----gcgatgg-----
SEQ ID NO:1	232 ttgaaacgcgcacatcatcggtcactggcagactgtaccggc-tatcggtg
SEQ ID NO:3	226 gtgaaatgggcattatgtggtcactgg-ggacaatcgccgcgtatttctg
SEQ ID NO:4	208 ctcatccaagctatca-caaaaactggtcaa-----aaaggtc
SEQ ID NO:5	41 -----aattacatgatgga---ga-tattgtta
SEQ ID NO:1	281 aactggctgtcgaaaacaagattgaagcttacaacttctcgagggcagc
SEQ ID NO:3	275 aactcgagaacaaaaataaattattgcttataactaccacaagggtga
SEQ ID NO:4	245 ttacatgtgtatcaacaatgcgggagttgataatt-----ggggac-
SEQ ID NO:5	65 atctcggg-----attg--gtttac-----caacacagg
SEQ ID NO:1	331 ttggtccactggttccgcgccttggcagggtcataagctcggcgtcttcac
SEQ ID NO:3	325 cttacacaaaccttacgcgcgcgcgcagcccaccagcctggtattattag
SEQ ID NO:4	287 ttggttgcctcttc--aaactcgacaaatc--aagaaaatgatctcctc
SEQ ID NO:5	92 ttgt-----taattatttacctgataatgtcaata-----ttac
SEQ ID NO:1	381 cgacatcggtct---ggaaa---ctttcctcgatcccgctcagctcggc
SEQ ID NO:3	375 tgatattggcat---cgggg---catttgctgatccacgccagcaaggc
SEQ ID NO:4	333 gtacgtcggtgaaaacggaga---atttgctcga---caatatcttagc
SEQ ID NO:5	126 --acttcaatca---gaaaatggcttcttggtttaactgca-----
SEQ ID NO:1	424 ggcaagctcaatgacgtaacca-----aagaagacctcgtaaaactgat
SEQ ID NO:3	418 ggcaaaactgaatgaagtcacta-----aagaagacctgattaaactggt
SEQ ID NO:4	376 ggagagctcgagttggaattcacaccacaaggaacactcgccgaacgaat
SEQ ID NO:5	163 -----tttgac---cca-----gaaaatgctaattcaaaact---
SEQ ID NO:1	468 cgaagtcgatgggtca---tgaacagcttttctaccggacc-----
SEQ ID NO:3	462 cgagtttgataacaa---agaatatctctattacaaagcg-----
SEQ ID NO:4	426 tcgtgcagctggtgccggtgttccgcattctacac-accacagggtac
SEQ ID NO:5	191 --tagtaaatgctgg---tggtcagcctt-----

SEQ ID NO:1	505	--ttcccgg--tcaacgtagctttcctccgcggtacgtatgctga---tg
SEQ ID NO:3	499	--attgcgc--cagatattgccttcattcgcgctaccacctgcga---ca
SEQ ID NO:4	475	ggtacccagattcaagaaggaggtgctccga--ttaagtacagtaaaactg
SEQ ID NO:5	215	-----gtggaa-----ttaa---aa
SEQ ID NO:1	548	aatccggcaatatac-accatggacg-----aagaaatcgggcctttc
SEQ ID NO:3	542	gtgaaggctacgcc-acttttgaag-----atgagggtgatgtatctc
SEQ ID NO:4	524	aaaaaggaaagattgaagttgcaagtaaaagcgaaagaacacgacaattc
SEQ ID NO:5	227	aaggcggtctta-----ctttt
SEQ ID NO:1	589	ga---aagcacttccgta---gccaggccggttcac--aactgtggcggt
SEQ ID NO:3	583	ga-----cgattggttattgccaggcggtgcac--aataacggcggt
SEQ ID NO:4	574	aatggaattaattatgtaaatggaaggctatttggggagattttgcatt
SEQ ID NO:5	244	ga---tagtgctt-----t--ttctttcgctt
SEQ ID NO:1	631	aaagtcgtcgtccagggtcaaagacgtcgtcgc-----tcacggcagcctc
SEQ ID NO:3	625	attgtgatgatgcagggtgcagaaaatggttaa-----gaaagccacgctg
SEQ ID NO:4	624	gatcaaggcgtggagagcagatac-tcttggaatatattcaattcagacat
SEQ ID NO:5	267	aa-----ttc
SEQ ID NO:1	676	gacccgcgcgatggtaagatccctg-----gcatctatgtogactac
SEQ ID NO:3	670	catcctaaatctgtccgtattccgg-----g---ttatctgggtggat
SEQ ID NO:4	673	.gctgctggaaatttcaataatccaatgtgcaaaagcctctaataatgcac--c
SEQ ID NO:5	272	gtggcggtcatgtt---gatgcctg-----tgtgctagggtggact--
SEQ ID NO:1	718	gtcgtcgtagcagctccggaagaccatcagcag--acgtatgactgcgaa
SEQ ID NO:3	709	attgtggtggtcgatccg---gatcaaacccaa--ctgtatggcggtgca
SEQ ID NO:4	721	atcgtcgaagtag---aggaaatcgtcgaaccgggagtaattgtccaaa
SEQ ID NO:5	309	-----
SEQ ID NO:1	766	t-----acgatccgtccctcagcggtgaacatcgtgtcctg-aaggc
SEQ ID NO:3	754	c-----cggttaaccgctttatttctggtgacttcacccttg-atgac
SEQ ID NO:4	768	cgatgtgcacattccatcaatctattgtcatcgtctagtgttgggaaga
SEQ ID NO:5	309	-----tg-aagtt
SEQ ID NO:1	808	gctac-----cgatgcagc-----tctccccatgagcgctaaga
SEQ ID NO:3	796	agtac-----caaacttag-----cctgccctaatac-caacgt
SEQ ID NO:4	818	actacaaaaaaccaatcgaaacggccaatgttcgcacacgaaggaccaata
SEQ ID NO:5	316	gatca-----agaagcaaa-----tctcgc-----
SEQ ID NO:1	842	aaatcatcggc-cgcccgcgcgctttggaattgactgaaaacgctgtcgt
SEQ ID NO:3	829	aaattagttgcgcggcgcgcatatttcgaaatgcgtaaaaggcggtggg
SEQ ID NO:4	868	aaaccatctac-atcggc--tgctggaaaatcgagagaaatcattg-cag
SEQ ID NO:5	336	-----taactgga-----
SEQ ID NO:1	891	caacctcggcgtcgggtgctcc-----ggaat--acgttgcttctgttgcc
SEQ ID NO:3	879	gaatgtcggcgctcggtattgc-----tgacg--gcattggcctggcgcc
SEQ ID NO:4	914	cacgtgcagctttggagttcacagatggaatgtacgccaatttgggtatc
SEQ ID NO:5	344	-----tggtgcc
SEQ ID NO:1	934	gg--tgaagaaggatcgccga-----tacca-----ttaacctgac
SEQ ID NO:3	922	cg--agaagaaggttgtgctga-----tgact-----ttattctgac
SEQ ID NO:4	964	gggattccgactttggcgccaaattatataccaaatggatttactgttca
SEQ ID NO:5	351	tg--gcaaaatggtgta-----
SEQ ID NO:1	969	cgtcgaagggtg-----gcgccatcggtggcgt-accgcagggcggtgcc
SEQ ID NO:3	957	ggtagaaacag-----gtccgattggcggaattacttcacaggggatcg
SEQ ID NO:4	1014	tttgcaaagtgagaatggtattattggagtggg-accata-----tcca
SEQ ID NO:5	364	-----

SEQ ID NO:1 1012 cgcttcggttcgtcccgcga-atgccgatgccatca----tcgaccacacc
SEQ ID NO:3 1001 c-ctttggcgcgaacgtga-ataccggtgccattc----tggatatgacg
SEQ ID NO:4 1057 agaaaag----gaacagaagacgccgatctcattaatgctggaaaagagc
SEQ ID NO:5 364 -----ccagga-atg-----

SEQ ID NO:1 1057 tatcagttcgacttctacgatggcggc-----ggtctggacatcg
SEQ ID NO:3 1045 tcccagtttgatttttatcacgggtggc-----ggtctggatggtt
SEQ ID NO:4 1103 ---caattactcttct-caaaggagcttcaattgttggttctgatgaatc
SEQ ID NO:5 373 -----ggcggg-----gcaatggacttag

SEQ ID NO:1 1097 cttacctcggcctgg-----cccagtgcgatg-----gctcgggcaac
SEQ ID NO:3 1085 gttatttgagttttg-----ctgaagtcgacc-----agcacggtaac
SEQ ID NO:4 1149 attcgcaatgattcgtggttctcatatggatattactgtgctcggtgcaac
SEQ ID NO:5 392 -----tg-----actggtgcaa-

SEQ ID NO:1 1135 atcaacgtcagca-agttcgggtactaacgttgccggctgcccgggtttcc
SEQ ID NO:3 1123 gtcggcgtgcata-aattcaatggtaaaatcatgggacccgggtggattta
SEQ ID NO:4 1199 ttca--gtgctcacagtttg-----agatttagcgaattggatgattcgg
SEQ ID NO:5 404 -----

SEQ ID NO:1 1184 ccaacatt--tcccagcagacaccgaatgtttacttctgcccgcacct-tc
SEQ ID NO:3 1172 ttgatatacgtgcccacttccaagaaaatcatt--ttctgcccgcacat-ta
SEQ ID NO:4 1243 ggaaaatt-----ggtga-aaggaatggcgggtgcaatggatcttgct
SEQ ID NO:5 404 -----aaaaagtgattatt-----ggca-----

SEQ ID NO:1 1231 acggctggcggcttgaaaatcgctgtcgaagacggcgaagtcgaatccct
SEQ ID NO:3 1219 actgcccggcagtttaaaacagaaattaccgacggcgaatataatccgt
SEQ ID NO:4 1285 tctgctcccgg-----agcccgtgt-gatcgttgtaatggagcatgtat
SEQ ID NO:5 422 -----tggaacattg-----tgccaagtcaggttccct

SEQ ID NO:1 1281 ccaggaaggcaagccaagaagttcatcaagctgtcgaccagatcactt
SEQ ID NO:3 1269 ccaggaaggacgggtgaagaaatttattcgggaactaccggaaattactt
SEQ ID NO:4 1328 cgaagaacggagagccaaaatt-----ctagagcactg
SEQ ID NO:5 449 caaaaattctaaag---aaatgtacattaccgct-----cacagcaagt

SEQ ID NO:1 1331 tcaacgg-----ttcctatgcagc---ccgcaacggcaaacacgttctct
SEQ ID NO:3 1319 tcagcggaaaaatcgctctcgagc---gagggctgg-----atgttcgtt
SEQ ID NO:4 1362 cgaac-----ttcctctga--c---cggcaaaagg--agtaatttcccg
SEQ ID NO:5 490 aaaaaag-----ttgccatggtggttaccgaattggca-----gtattta

SEQ ID NO:1 1373 a--catcacagaacgctgcgtatttgaactgacca--aagaa-ggcttga
SEQ ID NO:3 1361 a--tatcactgagcgcgcagtttccagctgaaag--aagac-ggcttgc
SEQ ID NO:4 1398 aatcattactgatattggcagttttcgacgtggacacaaagaacggattga
SEQ ID NO:5 530 a--cttcattgaaggcagattagttcta-----a--aagaa---catgc

SEQ ID NO:1 1418 aactcatcgaagtcgcaccgggcatcgatattgaaaaagatatcctcgct
SEQ ID NO:3 1406 atttaatacgaatcgcccctggcgtcgatttacaaaaagatatctcgac
SEQ ID NO:4 1448 cattgatcgaagt--caggaaggatc-ttactgtagatgatat-----
SEQ ID NO:5 567 tctcat-----gtggatttagaaaca---attaaagcc

SEQ ID NO:1 1468 cacatggacttcaagccgat--cattgata--atocga--aactcatgg
SEQ ID NO:3 1456 aaaatggatttcaccccagt--gatttcgcagaactca--aactgatgg
SEQ ID NO:4 1488 --caagaaactca--ccg-----cttgcaa--attcga--aatttccga
SEQ ID NO:5 598 aaaacag-----aagccgatttcattggt-----gccgatgatttcaaag

SEQ ID NO:1 1511 atgcccgcctcttccaggacgggtcccatggga-----ctgaaaaaa---
SEQ ID NO:3 1502 acgaaagattatttatcgatgcggcgatgggttttgcctgcctgaagcg
SEQ ID NO:4 1524 aaatctgaagccaatgggacaggtcctctta-----atcaaggataa-
SEQ ID NO:5 638 aaatgcaaatcagccag-----aaagga-----cttgaattatga

SEQ ID NO:1	1552	-----taa
SEQ ID NO:3	1552	gctcattaa
SEQ ID NO:4	1567	-----
SEQ ID NO:5	673	-----

Figure 9

SEQ ID NO:2	1	-----mrkveilit-----aeqaaqlv
SEQ ID NO:6	1	-----mpvls-----aqeavnyi
SEQ ID NO:7	1	mpilskiwaapaagilrktprnahqmrlismtssmkakvfnsaeaeavkdi
SEQ ID NO:8	1	-----mnakeli-----arriamel
SEQ ID NO:2	17	kdndtitsigfvssahpealt--kalekrfldtntpqnltyiyagsqgkr
SEQ ID NO:6	14	pdeatlcvlg-agggileattlitaladkykqtqtpnrnsiisptglgdr
SEQ ID NO:7	51	pdnakllvggfglclgipenli--qai-----tktgqkgtcvsnnagv-
SEQ ID NO:8	16	hdgd-ivnlg-----
SEQ ID NO:2	65	dgraaehlahtgllkraiighwqtvpaignklavenkieaynfsqgtlvhw
SEQ ID NO:6	63	adrgisplaqeglvkwalcghwggspriselaeqnkiiaynypqgvltqt
SEQ ID NO:7	92	dnwglglllqtrqikkmissyvgengefarqylsgeleleftpqgtlaer
SEQ ID NO:8	25	-----
SEQ ID NO:2	115	fralaghklgvftdigletfldprqlggklnvdkedlvkliev-----
SEQ ID NO:6	113	lraaaahqpgiisdigigtfdvprqggklnvdkedliklvef-----
SEQ ID NO:7	142	iraagagvpafytpgtgygtqi---qeggapikysktekgk-ievaskake
SEQ ID NO:8	25	-----igl-----
SEQ ID NO:2	159	----dgheqlfyptfpvnvafirgtyadesgnitmdeeigpfestsavaq
SEQ ID NO:6	157	----dnkeylyykaiapdiafirattcdseggyatfedevnyiyladalviaq
SEQ ID NO:7	188	trqfnginyvmeeaiwgdfalikawradtlgnlqfrhaagnfnnpckas
SEQ ID NO:8	28	-----ptqvv-----ylpdvnitlgsengflglta----
SEQ ID NO:2	205	vhncggkvvvqvkdvvahgsldprmvkipglyvdyvvvaapedhqgtydc
SEQ ID NO:6	203	vhnnggivmmqvqkmvkkatlhpksvripgyld-ivvdpdqgtqlygga
SEQ ID NO:7	238	--kc---tiveveeivepgviapndvhipsiychrlvlg-----knykk
SEQ ID NO:8	55	-----
SEQ ID NO:2	255	eydpslsgehrapegatdaalpsakkiigrrgaleltenavvnlgvg--
SEQ ID NO:6	252	pvnrfisgdftl-ddstklslplnqrklvarralfemrkavgnvgvg--
SEQ ID NO:7	277	pierpmfahegpikpstsaa--gksreiiaaraaleftdgmyanlgigip
SEQ ID NO:8	55	-fdp-----enansnl-vn--
SEQ ID NO:2	303	--apeyvasvageegiadtitltveggaig--gvpqggarfgssrnad--
SEQ ID NO:6	299	--iadgiglvareegcaddfiltvetgpig--gitsqgiafganvntr--
SEQ ID NO:7	325	tlapnyipn-----gftvhlqsengiigvgpyprkgtedadlinagke
SEQ ID NO:8	67	--a-----ggqpc--gikkggstf-----
SEQ ID NO:2	347	-----aiidhtyqdfdydgggldiaylglaqcdgsgni-nvskfgtn
SEQ ID NO:6	343	-----aildmtsqsdfyhgglldvcylsfaevdqhgnv-gvhkfnkg
SEQ ID NO:7	368	pitllkgasivgsdesfamirgshmditvlgalcscsfqgdlanwmipgkl
SEQ ID NO:8	82	-----dsafsfalirgghvdacvlgglevdeqeanianwmvpgkm
SEQ ID NO:2	388	vagcgggfpnisqgtpnvfyfcgtftagglkiav-----edgkvkilqegk
SEQ ID NO:6	384	imgtgffidisatskklifcgtltaglktei-----tdgklnivqegr
SEQ ID NO:7	418	vkmgggamdl-----vsapgarvivvmehvskngepkilehce
SEQ ID NO:8	121	vpgmggamlvtgakkvii-----gmehca-----ksgsskilk---
SEQ ID NO:2	432	akkfikavdqitfngsyaarnghvl--yitercvfel-tkegkklieva
SEQ ID NO:6	428	vkkiirelpeitfsgkialergldvr--yiteravftl-kedglhlhiea
SEQ ID NO:7	456	-----lpltgkgvisriitdmavfdvdtknlgltlievr
SEQ ID NO:8	155	-----kctlplt-----askkvam--vvtelavfnf-iegrlvlkeha

SEQ ID NO:2	479	pgidiekdi--lahmdfkpiidnp-klmdarlfqdgpmglk-----
SEQ ID NO:6	475	pgvdlqkdi--ldkmdftpvispelklmderlfidaamgfvlpaaah
SEQ ID NO:7	489	kdltvd-dikkltackfe-isenl-kpmgqaplnqg-----
SEQ ID NO:8	190	phvdle-ti--kakteadfivad-----dfkemqisqkglel-----

Figure 10

GTGAAACTGTGTATACTCTCGGAATCGAGGTTGGTTCTTCTTCTTCCAAGGCAGTCATC
CTGGAAGATGGCAAGAAGATCGTCGCCCATGCCGTCGTTGAAATCGGCACCGGTTGACC
GGTCCGGAACGCGTCCTGGACGAAGTCTTCAAAGATACCAACTTAAAAATTGAAGACATG
GCGAACATCATCGCCACAGGCTATGGCCGTTTCAATGTCGACTGCGCCAAAGGCGAAGTC
AGCGAAATCACGTGCCATGCCAAAGGGGCCCTCTTGAATGCCCCGGTACGACGACCATC
CTCGATATCGGCGGTGAGGACGTCAAGTCCATCAAATTGAATGGCCAGGGCCTGGTCATG
CAGTTTGCCATGAACGACAAATGCGCCGCTGGTACGGGCCGTTTCCCTCGACGTCATGTCG
AAGGTACTGGAAATCCCATGTCTGAAATGGGGGACTGGTACTTCAAATCGAAGCATCCC
GCTGCCGTCAGCAGTACCTGCACGGTTTTTGCTGAATCGGAAGTCATTTCCCTTCTTTCC
AAGAATGTCCCGAAAGAAGATATCGTAGCCGGTGTCATCAGTCCATCGCCGCCAAAGCC
TGCGCTCTCGTGCGCCGCGTCGGTGTCGGTGAAGACCTGACCATGACCGGCGGTGGCTCC
CGCGATCCCGGCGTCGTCGATGCCGATCGAAAGAATTAGGTATTCCTGTCAGAGTCGCT
CTGCATCCCCAAGCGGTGGGTGCTCTCGGAGCTGCTTTGATTGCTTATGATAAAATCAAG
AAATAA (SEQ ID NO:9)

Figure 11

VKTVYTLGIDVGSSSSKAVILEDGKKIVAHAVVEIGTGSTGPERVLDEVFKDTNLKIEDM
ANIIATGYGRFNVDCAKGEVSEITCHAKGALFECPGTTTTILDIGGQDVKSIKLNQGLVM
QFAMNDKCAAGTGRFLDVMSKVLEIPMSEMGDWYFKSKHPAAVSSTCTVFAESEVISLLS
KNVPKEDIVAGVHQSIKACALVRRVGVGEDLTMTGGGSRDPGVVDAVSKELGIPVRVA
LHPQAVGALGAALIAYDKIKK (SEQ ID NO:10)

Figure 12

SEQ ID NO:9	1	gtgaaaactgtgtatactctcggaatcgacgttggttcttcttcttccaa
SEQ ID NO:11	1	---atgagtatctataccttggaatcgattggtatctactgcatocaa
SEQ ID NO:12	1	gtggcagtgggcatattcgattggcattgattccggctcaaccggccacaa
SEQ ID NO:13	1	-----atgattttagggatagatgttgatctacaacaacgaa
SEQ ID NO:9	51	ggcagtcacacctggaagatggcaagaagatcgtcgc-ccatgccgtcggt
SEQ ID NO:11	48	gtgcattatcctgaaagatggaaaaaagaatcgtagc-gaaatccctggta
SEQ ID NO:12	51	agggatcttactggcagacggcggtgatta----cgcgccgttctctcggt
SEQ ID NO:13	39	gatggttctaattggaagatagc---aagataatttg-gtataagatagag
SEQ ID NO:9	100	gaaatcggcaccgggttcgaccgggtccggaacgcgtcctggacgaagtctt
SEQ ID NO:11	97	gccgtggggaccggaaacttccgggtcccgcacgggtctatttcggaagtcct
SEQ ID NO:12	97	ccaa----ccccctttcgcccg-gaacagcaattact----gaagcctg
SEQ ID NO:13	85	gatattgg-agttgtta-----ttgaggagatattttattaaaaatggt
SEQ ID NO:9	150	caaagatacc-aactttaaaaattgaagacatggcgaacatcatcgc-cac
SEQ ID NO:11	147	ggaaaatgcc-cacatgaaaaagaagacatggcctttaccctggc-tac
SEQ ID NO:12	138	ggaa-actct-gcgcgaagggttagagacaacgccgtttctgacgctcac
SEQ ID NO:13	129	taaggagattgaacaaaaatatccaatagat----aaaatcgttgc-aac
SEQ ID NO:9	198	aggetatggcgggttcaatgtcg-----actgcgccaaaggcggaag
SEQ ID NO:11	195	cggtacgggacg---caat-tcgttggaaggcattgccgacaagcaga--
SEQ ID NO:12	186	cggtacggggcggaactggtgg-----atgttccgataaaacagg
SEQ ID NO:13	174	tggatatggaaggcataaggtta-----gttttcgagataagatag
SEQ ID NO:9	239	tcagcgaaatcacgtgccatgccaaaggggcc---ctctttgaatgccccc
SEQ ID NO:11	239	tgagcgaaactgagctgccatgccatggggcgcc---agctttatctggccc
SEQ ID NO:12	227	taacggaaatctcctgtcacgggctggggcgca---cggtttcttgcgcca
SEQ ID NO:13	215	ttccagaagtta-ttgcattgggaaaaggagctaactatttctttaacga
SEQ ID NO:9	286	ggtacgacga--ccatcctcgatatcgggcggtcaggacgtcaa-gtccat
SEQ ID NO:11	286	--aacgtccataccgtcatcgatatcgggcgggcaggatgtgaa-ggtcat
SEQ ID NO:12	274	gcaacgcgcg--cggtaatcgacatcggtggtcaggacagcaaagtgtt
SEQ ID NO:13	264	ggcagatgga----gttatagacattggagggcaagatacaaaa-ggtctt
SEQ ID NO:9	333	caaattga--atggccagggcctggtcatgcagtttgcc-atgaacgaca
SEQ ID NO:11	333	ccatgtgg--aaaacggggaccatgacca---atttccag-atgaatgata
SEQ ID NO:12	322	cagcttgatgatgacggtaacctg----tgcgatttctctgatgaatgaca
SEQ ID NO:13	309	aaagattg--ataaaaacggaaaagtgttgattttatc-ctatcagata
SEQ ID NO:9	380	aatgcgccgtggtacggggcggtttcctcgacgtcatgtcgaaggtactg
SEQ ID NO:11	377	aatgcgctgccgggactggcggtttcctggatgttatggccaatatcctg
SEQ ID NO:12	368	aatgcgcggcgggcaccggcggtttcctggaggtgatctcgcgacgctt
SEQ ID NO:13	356	aatgtgccgctggaactggaaaattcttaga-----aaaggcatta
SEQ ID NO:9	430	gaaatcccatgtct-ga--aatgggggactggtactt-caaatcgaagc
SEQ ID NO:11	427	gaagtgaagggtttcc-ga--cctggctgagctgggagc-caaatccacca
SEQ ID NO:12	418	ggca--ccagcgtcgagc--aactcgacagcattaccg-aaaat---gtc
SEQ ID NO:13	397	gatattttaaaaatt-gataaaaatgagataaataaatacaaatcagata
SEQ ID NO:9	476	atcccgt-gccgtcagcagctacctgcacgggtttttgctgaatcggaggt
SEQ ID NO:11	473	aacgggtg-gctatcagctccacctgtactgtgtttgcagaaagtgaagt
SEQ ID NO:12	460	acgcgcacgccatcacgagtatgtgcacagtggttgcgtgaatcagaagc
SEQ ID NO:13	446	atatcgct-aaaatatctcaatgtgtgctgtcttctgctgaaagtgaagt

SEQ ID NO:9	525	catttcaccttctttccaagaatgtcccgaagaa--gatatcgtagccgg
SEQ ID NO:11	522	catcagccagctgtccaa--aggaaccgacaagatcgacatcattgccgg
SEQ ID NO:12	510	gatcagcctgcgctcagcgggctcgcgccagaa--gcgattctgcagg
SEQ ID NO:13	495	aataagcttactatcaaaaaaagtccaaaggaa--ggcattttaatggg
SEQ ID NO:9	573	tgtccatcagtcocatcgccgccaaagcctgcgctctcgtgc-gccgcgtc
SEQ ID NO:11	570	gatccatcgttctgtagccagccgggtcattggtcttgcca-atcgggtg
SEQ ID NO:12	558	agtgattaacgcgat-ggcgcggaggagtgc-caatttcat-tgctcgtc
SEQ ID NO:13	543	cgctctatgagagtat-----aataaatagggttatcccaatgaccaata
SEQ ID NO:9	622	ggtgtcgg--tgaagacctgaccatgaccggcggtggtccccgcgat--c
SEQ ID NO:11	619	gggattgt--gaaagacgtggtcatgaccggcggtgtagcccagaac--t
SEQ ID NO:12	605	tctc-ctg--tgaagcgcgattctgtttactgggtggcgttagtcattgc
SEQ ID NO:13	587	ggcttaaaattcaaaacatagtgtttagtggaggagtgtgctaaaaat--a
SEQ ID NO:9	668	ccggcgtcgtcgatgccgtatcgaaagaat-----taggtattcctgtc
SEQ ID NO:11	665	atggcgtgagaggagccct-----ggaag-----aaggccttggcgtg
SEQ ID NO:12	652	cagaagt-----ttgcccgatgctggaatctcacctgcgaatgccggta
SEQ ID NO:13	635	aggttttggttgagatgtttgagaaaaaat-----tgaataaaaaacta
SEQ ID NO:9	712	agagtcgctctgcatccccaagcgggtg-----ggtgctctcggagctgc
SEQ ID NO:11	703	gaaatcaagacgtctccctggctcagtacaacgggtgccctgggtgccgc
SEQ ID NO:12	697	aatacccatcctgatgcgcaatttgct-----ggcgcaattggcgcggc
SEQ ID NO:13	679	ctaattccaaaagaaccacagattgtt-----tgctgtgttgagctat
SEQ ID NO:9	756	tttgattgctta-----tgataaaatcaagaaa-taa
SEQ ID NO:11	753	tctgtatgcgta-----t-aaaaaagcagccaaataa
SEQ ID NO:12	741	ggttaattggtcaacgagtgaggacacgccgatga---
SEQ ID NO:13	723	attggtt-----taa-----

Figure 13

SEQ ID NO:10	1 vktvytlgidvgsssskaviledgkklivahavveigtgstgpervldevf
SEQ ID NO:14	1 ms-lytlgidvgstaskciilkdgkeivakslvavgtgtsgparsisevl
SEQ ID NO:15	1 mavaysigidsgstatkgilladg-vitrflvpt---pfrpataiteaw
SEQ ID NO:16	1 ----milgidvgstttkmvlmeds-kiiwykiedigv---viedillkmv
SEQ ID NO:10	51 kdtlnkiedmaniatgygrfnvd-cakgevseitchakgalfecpgttt
SEQ ID NO:14	50 enahmkkedmaftlatgygrnslegiadkqmselschamgasfiwpnvht
SEQ ID NO:15	47 etlreglettpfltltygygrqlvd-fadkqvteischglgarflapatra
SEQ ID NO:16	44 keieqkyp-idkivatgygrhkvs-fadkivpevialgkanyffneadg
SEQ ID NO:10	100 ildiggqdvksiklngqglvmqfamndkcaagtgrfldvmskvleipmse
SEQ ID NO:14	100 vidiggqdvkvihev-ngtmtntfqnmdkcaagtgrfldvmanilevkvsd
SEQ ID NO:15	96 vidiggqdsqviqldddgnlcdfilmndkcaagtgrflevisrtlgtvsveq
SEQ ID NO:16	92 vidiggqdtkvkldkngkvvdflisdkcaagtgkflekaldilkidkne
SEQ ID NO:10	150 mgdwyfkskhpaavsstctvfaesevisllsknvpkedivagvhqsiaak
SEQ ID NO:14	149 laelgakstkrvaisstctvfaesevisqlskgtdkididiagihrsvasr
SEQ ID NO:15	146 l-dsitenvtphaitsmctvfaeseaislrsagvapeailagvinamarr
SEQ ID NO:16	142 ink--yksdniakissmcavfaeseisllskkvpkegilmgvyesiinr
SEQ ID NO:10	200 acalvrrvgvgedlmttgggsrdpgvvdavskelgipvrvalhpqavgal
SEQ ID NO:14	199 vigilanrvgivkdvvmtggvaqnygvrgaleeglgveiktsplaqyngal
SEQ ID NO:15	195 sanfiarlsceapilftggvshcqkfarmleshlrmpvnthpdaqfagai
SEQ ID NO:16	190 vipmntnrki-qnivfsggvaknkvlvemfekklkklipkepqiavccv
SEQ ID NO:10	250 gaaliaydkikk--
SEQ ID NO:14	249 gaalyaykkaak--
SEQ ID NO:15	245 gaavig-qrvrtrr
SEQ ID NO:16	239 gailv-----

Figure 14

ATGAGTGAAGAAAAACAGTAGATATTGAAAGCATGAGCTCCAAGGAAGCCCTTGGTTAC
TTCTTGCCGAAAGTCGATGAAGACGCACGTAAAGCGAAAAAGAAGGCCGCCTCGTTTGC
TGGTCCGCTTCTGTGCTCCTCCGGAATTCTGCACGGCTATGGACATCGCCATCGTCTAT
CCGGAAACTCACGCAGCTGGTATCGGTGCCCGTCACGGTGCTCCGGCCATGCTCGAAGTT
GCTGAAAACAAAGGTTACAACCAGGACATCTGTTCTACTGCCCGCTCAACATGGGCTAC
ATGGAACTCCTCAAACAGCAGGCTCTGACAGGCGAAACGCCGGAAGTCCTCAAAAACCTCC
CCGGCTTCTCCGATTCCCCTTCCGGATGTTGTCTCACTTGCAACAACATCTGCAATACC
TTGCTCAAATGGTATGAAAACCTGGCTAAAGAATTGAACGTACCTCTCATCAACATCGAC
GTACCGTTCAACCATGAATTCCTGTTACGAAACACGCTAAACAGTACATCGTCGGCGAA
TTCAAACATGCTATCAAACAGCTCGAAGACCTTTGCGGCCGTCCCTTCGACTATGACAAA
TTCTTCGAAGTACAGAAACAGACACAGCGCTCCATCGCTGCCTGGAACAAAATCGCTACG
TACTTCCAGTACAAACCGTCGCCGCTCAAGGGCTTCGACCTCTTCAACTACATGGGGCTC
GCCGTTGCTGCCCGCTCCTTGAACTACTCGGAAATCACGTTCAACAAATTCTCAAAGAA
TTGGACGAAAAAGTAGCTAATAAGAAATGGGCTTTGGGTGAAAACGAAAAATCCCGTGTT
ACTTGGGAAGGTATCGCTGTCTGGATCGCTCTCGGCCACACCTTCAAAGAACTCAAAGGT
CAGGGCGCTCTCATGACTGGTTCCGCTTATCCTGGCATGTGGGACGTTTCTACGAACCG
GGCGACCTCGAATCCATGGCAGAAGCTTATCCCGTACATACATCAACTGCTGCCTCGAA
CAGCGCGGTGCTGTTCTTGAAAAAGTTGTCCGCGATGGCAAATGCGACGGCTTGATCATG
CACCAGAACCGTTCTTGCAAGAACATGAGCCTCCTCAACAACGAAGGCGGCCAGCGCATC
CAGAAGAACCTCGGCGTACCGTACGTCATCTTCGACGGCGACCAGACCGATGCTCGTAAC
TTCTCGGAAGCACAGTTGGATAACCGCGTAGAAGCTTTGGCAGAAATGATGGCAGACAAA
AAAGCCAATGAAGGAGGAAACCACTAA (SEQ ID NO:17)

Figure 15

MSEKTVDIESMSSKEALGYFLPKVDEDARKAKKEGRLVCWSASVAPPEFCTAMDIAIVY
PETHAAGIGARHGAPAMLEVAENKGYNQDICSYCRVNMGYMELLKQQALTGETPEVLKNS
PASPIPLPDVVLTCNNICNTLLKWIENLAKELNVPLINIDVPFNHEFPVTKHAKQYIVGE
FKHAIKQLEDLCGRPFDDYDKFFEVQKQQTORSIAAWNKIATYFQYKPSPLNGFDLFNYMGL
AVAARSLNYSEITFNKFLKELDEKVANKKWAFFGENEKSRTWEGIAVWIALGHTFKELKG
QGALMTGSAYPGMWDVSYEPGDLESMAEAYSRTYINCCLEQRGAVLEKVVRDGGKCDGLIM
HONRSCKNMSLLNNEGGQRIQKNLGVPIVIFDGDQTDARNFSEAQFDTRVEALAEMMADK
KANEGGNH (SEQ ID NO:18)

Figure 16

SEQ ID NO:17	1 atgagtgaagaaaaaacagtagatattgaaagcatgagctccaaggaagc
SEQ ID NO:19	1 atg-----ccaaagacagta-----agccctggcggttcagg----
SEQ ID NO:20	1 ----atgatgaaattaaag--gcaattgaaaagttga--tgcaa-----
SEQ ID NO:21	1 -----atgtcacttgtcaccga-----tcta--cccg
SEQ ID NO:17	51 cctt---ggttacttcttgccgaaa--gtcgatgaagacgca-----c
SEQ ID NO:19	32 -cat---tgagagatgtagttgaaaagggttacagagaactg-----c
SEQ ID NO:20	37 -----aaatt-----cgcca--gtagaaaagaacagc-----t
SEQ ID NO:21	27 cattttcgatcagttct--ctgaag--ctcgccagacagggtttctcacc
SEQ ID NO:17	89 gta-aagcgaaaa-aagaaggccgctcggtt--gctgggtccgcttctgtc
SEQ ID NO:19	71 ggg-aaccgaaaag--aaagaggagaaaaagtag-gctgggtcctcttc--ca
SEQ ID NO:20	63 atataagcaaaaaagaagaaggtagaaaagttt--ttggaatgttctgtg
SEQ ID NO:21	73 gtc-atggatctc-aaggag--cgcggtcattccgctggt-----tggc
SEQ ID NO:17	136 gtcctcctcggaattctgcacggctatggacatcgccatcgctc--tatccg
SEQ ID NO:19	116 agttccctgcgaactggctgaatcttttcgggtgcatgttgggtatccg
SEQ ID NO:20	110 cct-----atgttcca-----atagaaat--aat--tt--tagcag
SEQ ID NO:21	112 act-----tactgcacctttatg----ccgcaagag-----atccc
SEQ ID NO:17	184 gaaactca--cgagctgggtatcggtgcc--cgtcacgggtg-----
SEQ ID NO:19	166 gaaaacca--ggctgctgggtatcgctgccaaccgtgacggcggaagtgatg
SEQ ID NO:20	140 caaatgcaatcccagttggttgtgtgga--ggtaaaaat-----
SEQ ID NO:21	144 ga-----t--ggcagc-----cggtgcg--gtt--gtg-----
SEQ ID NO:17	221 -----ctccggccatgc
SEQ ID NO:19	214 tgccaggctgcagaagatatcggttatgacaacgatatctgcggctatgc
SEQ ID NO:20	178 -----gacacaa
SEQ ID NO:21	166 -----gttcgctctgt
SEQ ID NO:17	233 tcgaagt-t-----gctg-----aaaa--
SEQ ID NO:19	264 ccgtatt-tccctggcttatgctgccgggttcgggggtgccaaacaaatg
SEQ ID NO:20	185 tcccaat-a-----gcag-----a-----
SEQ ID NO:21	178 tccacctct-----gatg-----aaac--
SEQ ID NO:17	249 --caaaggttacaaccaggacatctgttctactgcccgtcaacatg--
SEQ ID NO:19	313 gacaaagatggcaactatgtcatcaacccccacagcgggcaaacagatgaa
SEQ ID NO:20	198 --ggaggat-ttgccaagaaacctatgcc-----cattaata----
SEQ ID NO:21	195 --ca---ttgaagaagcggagaaagat----ctgccgcg-caacct--
SEQ ID NO:17	295 -----ggctacatggaactc--ctcaaacagcag-----
SEQ ID NO:19	363 agatgccaatggcaaaaagggtattcgacgcagatggcaaacccgtaatcg
SEQ ID NO:20	232 -----aaatc--atccta--tg-----
SEQ ID NO:21	231 -----ctgcccg--ctg--attaaa-agca-----
SEQ ID NO:17	322 -----
SEQ ID NO:19	413 atcccaagaccctgaaaccctttgccaccaccgacaacatctatgaaatc
SEQ ID NO:20	245 -----
SEQ ID NO:21	251 -----
SEQ ID NO:17	322 ---gctctgac---aggcgaaa-----cgccggaa-gtcctcaa
SEQ ID NO:19	463 gctgctctgccggaagggaagaaaagacccgccgcagaatgccttgca
SEQ ID NO:20	245 ---gttttaa-----gaa-ggca--aa
SEQ ID NO:21	251 ---gtacggc---ttcgga--aaccg-----at

SEQ ID NO:17 354 aaactccccggcttctccgattccccctccggatgttgctcacttgca
SEQ ID NO:19 513 caaatatcgtcagatgacatgccatgccggacttcgtgctgtgctgca
SEQ ID NO:20 261 aacctgccc--ttactttgaagcatct----gatatagttat-tggagaa
SEQ ID NO:21 274 aaatgcccctacttct-----acttttcggatctcgttggtc---ggtg

SEQ ID NO:17 404 acaacatctgca-----ataccttgctcaaattggtatgaaaacttg-
SEQ ID NO:19 563 acaacatctgca-----actgcatgaccaaattggtatgaagacattg-
SEQ ID NO:20 304 actacctgtgaaggaaagaagaagatgtttgagttgatggagagattggt
SEQ ID NO:21 314 aaaccacctgcg-----acggcaaaaaaagaattgtatgaatacatgg-

SEQ ID NO:17 446 -ctaaagaattgaac---gtacctctca---tcaacatcgacgtac--c
SEQ ID NO:19 605 -cccgtcggcacaac---attcctttga---tcatgatcgacgttc--c
SEQ ID NO:20 354 gccaatgcatataat---gcacctccacacatgaaagatgaagatt--c
SEQ ID NO:21 356 -c---ggagtttaagcctgttcatgtga---tgca-attgcccacagc

SEQ ID NO:17 486 gttca--accatgaattc---cctg--tta-cgaa----ac--acgctaa
SEQ ID NO:19 645 ttaca--ac---gaattcgaccatg--tcaacgaa----gccaacgtgaa
SEQ ID NO:20 399 tttga--a-----aatct---ggat--taa-agaagttgaa--aagctaa
SEQ ID NO:21 397 gttaaggacgatgcctcg---cgtgcgtta-tgga----a-----

SEQ ID NO:17 522 acagtacatcgctg-----gcgaattcaaacatgctatca----aacagc
SEQ ID NO:19 684 a---tacctccggt-----ccagctggatcggccatcc----gtcaaa
SEQ ID NO:20 434 --aagaattggttgagaaagagactggaaataaaataacagaggaaaagt
SEQ ID NO:21 429' --agccgagatgct-----gcgcttgcaa-----a-----aaacgg

SEQ ID NO:17 563 tcgaagacctttgcggccgctcccttcgactatgacaaattcttcgaagta
SEQ ID NO:19 722 tggaagaatcaccggcaagaagtgcgatgaagacaaattc-----gaa
SEQ ID NO:20 482 taaaaga-----gacagttgat--aaagta
SEQ ID NO:21 458 tagaagaacgttttgggcacgagattagcgaagatgctctgcgcgatgcc

SEQ ID NO:17 613 cagaaacagacacagcgctc-catcg--ctgcc-----tggaacaaaat
SEQ ID NO:19 766 cag-tgctgccagaacgc-c-aaccgtactgccaaagcatggctgaaggt
SEQ ID NO:20 505 aataaagttagggag-----t-----tgttttataaa
SEQ ID NO:21 508 attgcgctgaaaaaccgcgaacgtcg--cgcac-----tgg--ctaatt

SEQ ID NO:17 654 cgctacgtacttc--c--agtacaaaaccgtcgccgctcaacggcttcgac
SEQ ID NO:19 813 ttgcgactacctg--c--agtacaaaaccggctccggtcaacgggttcgac
SEQ ID NO:20 532 ctctatgaattga--ggaagaataaaaccagctccaattaagggttagat
SEQ ID NO:21 547 ttttatcatcttggtgc--agttaaatcctccggcgcttagcggcagcgac

SEQ ID NO:17 700 ctcttcaactacatgggcctcgccg--ttgctgcccgtccttgaactact
SEQ ID NO:19 859 ctgttcaaccatattggtgacgtgg--ttaccgcccgtggccgtgtggaag
SEQ ID NO:20 580 gttttaaaattattccagtttgccatttatttgatattgatgacacaat
SEQ ID NO:21 595 attctga---aagtggtttacggcg-caaccttccggttcgataaagagg

SEQ ID NO:17 749 cggaatcacgttcaacaaattcctcaaagaattggacgaaaaagtagc-
SEQ ID NO:19 908 ctgctgaagctttcgaactgctggccaaggaaactggaacagcatgt----
SEQ ID NO:20 630 agggatt----ttagaggatttaattgaggagttagaggagagagtt---
SEQ ID NO:21 641 cg-----ttgatcaatgaactggatgcaatgaccgcc

SEQ ID NO:17 798 -----taataagaaatgggctttcggtgaa-----aacgaaaaatcccg
SEQ ID NO:19 954 -----gaaggaaggcaccaccaccgctcccttcaaagaacagcatcg
SEQ ID NO:20 673 -----aaaaaaggagaaggttatgaaggaa-----agagaa-----
SEQ ID NO:21 673 cgcgttcgtcagcagtggaagaag--gcc-----agcgactggaccgg

SEQ ID NO:17 837 tgttacttggaaggta--tcgctgtctggatcgctctcggccacacc---
SEQ ID NO:19 996 tatcatgttcgaaggga--tccctgctgg--ccgaaactgccgaacc---
SEQ ID NO:20 704 -----ttttaataac-tggctgtc-caatgggttgctggaacaataag
SEQ ID NO:21 715 cgt--ccgcgcattttaatcacgggctg---cccgattggcggcgcc---

SEQ ID NO:17 883 ----t--tcaaagaactca--aaggtcagggcgctctcatgactgggttcc
SEQ ID NO:19 1040 ----tgttcaaaccgctga--aagccaacggcctgaacatcaccggcggtt
SEQ ID NO:20 745 attgt--tgaaattattgaggaagtt---ggaggagtagttggtggtgaa
SEQ ID NO:21 756 -----agcaga--aaaagtgggtgcgcgcgattgaagagaatg

SEQ ID NO:17 925 gcttat---cctggcatgtgggacgtttcctacgaacc-----ggg-
SEQ ID NO:19 1084 gtatatgctcctgcttctcggttcgtgtacaacaacct-----gga-
SEQ ID NO:20 790 g--aaa---gctgcactggaacaagattctttgaaaactttgttgaggg-
SEQ ID NO:21 791 gc---g---gctgggttgcgtgttatgaaaactgcacc-----gggg

SEQ ID NO:17 963 -----cga---cctcg-aatccatggcagaa----gcttattcccgtac
SEQ ID NO:19 1125 -----cga---attgg---tcaaagcctact----gcaaagccccgaac
SEQ ID NO:20 834 -----ctatagcgtag--aggacattgcaaaa----agata----cttta
SEQ ID NO:21 827 cgaaagcga---ccgagcaatgcgtggcagaaacgggcgatgtctacgac

SEQ ID NO:17 999 atac-----atcaactgctgcct-----cgaacagcgcggtgct
SEQ ID NO:19 1159 -tcc-----gtca-----gcat-----cgaacaggggtgttgcc
SEQ ID NO:20 869 aaat-----cccatgtgctttagatttaaaaacgatgagagagttgaa
SEQ ID NO:21 874 gcgctggcggataaatatctggc-----gattggctgctcct

SEQ ID NO:17 1033 gttcttgaaaaagtgtgccgcatggcgaatgcgcagggc--ttgatcatgc
SEQ ID NO:19 1186 tggcgtgaaggcctgatccgcgacaacaaggttgacggc-gtactgggttc
SEQ ID NO:20 913 aatataaagagattggttaaagagttggacgtcgatggagttgtttat--
SEQ ID NO:21 911 gtgtttcgcgaacgatcagcgcctgaaaatgc--tcagc--cagatggtgg

SEQ ID NO:17 1082 accagaacc-gttcctgcaagaacatgagcctcctcaacaacgaaggcg-
SEQ ID NO:19 1235 actacaacc-ggtcctgcaaacocctggagcgggtacatgcctgaaatgc-
SEQ ID NO:20 961 ----tacac-tttgcagtattgccat----acatttaacatagagggagc
SEQ ID NO:21 959 aggaatatcaggtcgatggcgtagttga----tgtgattttgcaggcgt

SEQ ID NO:17 1130 ---gccagcgcac--cagaagaacctc--ggcgtaccgtacgtcatcttc
SEQ ID NO:19 1283 ---agcgtcgtttc--accaaagacatg--ggtatccccactgctggattc
SEQ ID NO:20 1002 taaggtagaggagg--cattaaaagaggaggccattccaattataagaatt
SEQ ID NO:21 1004 ---gccatacctacgcgggtggaatcgc--tggcgattaaacgtcatgtgc

SEQ ID NO:17 1174 gacggcgaccagaccgatgctcgttaacttctcggaagca-----
SEQ ID NO:19 1327 gacggtgaccaggctgacccgagaaacttcaacgcggct-----
SEQ ID NO:20 1051 gaaactgactattctga-----aagtgatag--agag-----
SEQ ID NO:21 1049 gccagc-agcacaacattccttatatcgctattgaaacagactactccac

SEQ ID NO:17 1213 -----cagttcgatacccgcgtagaagctttggcagaaatga
SEQ ID NO:19 1366 -----cagtatgagaccggtgttcagggcttggtcgaagcca
SEQ ID NO:20 1081 -----cagttaaaaacaaggttgaggcatttattgagatga
SEQ ID NO:21 1098 ctcggtatgtcgggcagctcagtaaccggtgcgcggcctttattgagatgc

SEQ ID NO:17 1250 tggcagacaaaaaagccaatgaaggaggaaaccactaa
SEQ ID NO:19 1403 tggaaag-caaatgatgaaaagaagg-ggaaataa----
SEQ ID NO:20 1118 t-----ttaa-----
SEQ ID NO:21 1148 tgtaa-----

Figure 17

SEQ ID NO:18	1 --mseektvdiessmsskealgyflpkvdedarkakkegrlvcwsasvapp
SEQ ID NO:22	1 ----mpktvs----pgvqalrdvvekvyrelrepkergekvswssskfpc
SEQ ID NO:23	1 --mmklka--iekimqkfa-----srkeqlykqkeegrkvfgm-----
SEQ ID NO:24	1 mslvtdlpaifdqfsearqtg-fltvmdlkergiplvg-----
SEQ ID NO:18	49 efctamdlaivypethaag---igarhgapamleavenkgynqdicyscr
SEQ ID NO:22	43 elaesfrlhvgypenqaag---iaanrdgevmcqaadigyndicgyar
SEQ ID NO:23	35 -fcayvpieila-anaip---vglcggkndtipiae-edlprnlcpklik
SEQ ID NO:24	38 tyctfmpqei----pmaagavvsvlcsdsdetieeae-kdlprnlcpklik
SEQ ID NO:18	96 vnmgyam-----
SEQ ID NO:22	90 islayaagfrgankmdkdgnyvinphsgkqmkdangkkvfdadgkpvldp
SEQ ID NO:23	79 ssygf-----
SEQ ID NO:24	83 ssygf-----
SEQ ID NO:18	102 ellkqgaltgetpev-----lknspaspiplpdvvlctcn
SEQ ID NO:22	140 ktlkpfattdniyeiaaapegeektrrqnalhkyrqmtmpmpdfvlccnn
SEQ ID NO:23	84 -----kkactcpyfeasdiviget
SEQ ID NO:24	88 -----gktdkcpyf-----y-----fsdlvvg-et
SEQ ID NO:18	137 icntllkwyenlakelnvplnidvpfnhefpvtkhakqyivgefkhak
SEQ ID NO:22	190. icncmtkwyediarrhniplimidvpynefdhvneanvkylrsqldtair
SEQ ID NO:23	103 tceggkkmfelm--erlvpmhimhlphmkd----edslikiweveklke
SEQ ID NO:24	107 tcdgkkmeyeymaefkpvhvmqlpnsvkdd-----asralwkaemlrllqk
SEQ ID NO:18	187 qledlccgrpfdydkffe---vqkqtqrsiaawnkiatyfyqkpsplngfd
SEQ ID NO:22	240 qmeeitgkfkdedkfeq---ccqnanrtakawlkvcdylqykppafngfd
SEQ ID NO:23	147 lveketgnkiteeklke---tvdkvnkvrelfyklyelrknpapikgld
SEQ ID NO:24	152 tveerfgheisedalrdaialknrreralanfyhlg---qlnppalsgsd
SEQ ID NO:18	234 ---lfnymglavaarslseyseitfnkflkeldekvan--kkwafge--n-
SEQ ID NO:22	287 ---lfnhmadvvtargrveaaeafellakeleghvke--gtttapf--k-
SEQ ID NO:23	194 vlklfqfaylldiddttigile----dlieeleerv----kk--ge--gy
SEQ ID NO:24	199 ---ilk---vvygatfrfdk---ealineldamtarvrqqweegqrld-
SEQ ID NO:18	276 eksrvtwegiavwialghtfkelkgqgalmtg----say---pgmwdvsv
SEQ ID NO:22	329 eqhrimfegipcwplkplnkplkanglnitg----vvy---apafgfvy
SEQ ID NO:23	231 egkrilitgcpmvagnnkiveiieevggvvvg----eesctgtrffenfv
SEQ ID NO:24	238 prprilitgcpiggaekvvraieenggwvvgyenctga---kateqcva
SEQ ID NO:18	319 epgd1-esmaeaysrtyinccl--eqrgavlekvvrdgkcdglimhqns
SEQ ID NO:22	372 --nnl-dclvkayckapnsvsi--eqgvawreglirdnkvdgvlvhynrs
SEQ ID NO:23	277 egysv-ediakryfkipcacrfkndervenikrlvkelddvgvvytlqy
SEQ ID NO:24	285 etgdvydaladkylaigcscvspndqrlkmlsqmveeyqvdgvdvilqa
SEQ ID NO:18	366 cknmsllnnegg--griqknlgvpyvifdgdqtdarnfseaqfdtrveal
SEQ ID NO:22	417 ckpwsgympemq--rrftkdmgiptagfdgdqadprnfnaaqyetrvggl
SEQ ID NO:23	326 cht---fniegakveealkeegipiirietdyses---dreqlktrleaf
SEQ ID NO:24	335 chtyaveslaik--rhvrqqhnpypiai---etdystsdvgqlstrvaaf
SEQ ID NO:18	414 aemmadkkanegggh
SEQ ID NO:22	465 veameandekkgk--
SEQ ID NO:23	370 iemi-----
SEQ ID NO:24	380 ieml-----

Figure 18

ATGAGTCAGATCGACGAACTTATCAGCAAATTACAGGAAGTATCCAACCATCCCCAGAAG
ACGGTTTGAATTATAAAAAACAGGGTAAAGGCCTCGTAGGCATGATGCCCTACTACGCT
CCGGAAGAAATCGTATATGCTGCAGGCTACCTCCCGGTAGGCATGTTTCGGTTCCCAGAAC
CCGCAGATCTCCGCAGCTCGTACGTAGCTTCCTCCGTTTCGCTTGCTCCTTGATGCAGGCT
GACATGGAACTCCAGCTCAACGGCACCTATGACTGCCTCGACGCTGTTATCTTCTCCGTT
CCTTGCGACACTCTCCGCTGCATGAGCCAGAAATGGCACGGCAAAGCTCCGGTCATCGTC
TTCACACAGCCGCGAGAACCCTAAGATCCGCCCCGGCTGTGATTTCTCAAAGCTGAATAC
GAACATGTCCGTACGGAATTGGGACGTATCCTCAACGTAAAAATCTCCGACCTGGCTATC
CAGGAAGCTATCAAAGTATATAACGAAAACCGTCAGGTTATGCGTGAATTCTGCGACGTA
GCTGCTCAGTACCCGCGAGATCTTCACTCCGATAAAAACGTCATGACGTCATCAAAGCCCGC
TGGTTCATGGACAAAGCTGAACACACCGCTTTGGTCCGCGAACTCATCGACGCTGTCAAG
AAAGAACCGGTACAGCCGTGGAATGGCAAAAAGTCATCCTCTCCGGTATCATGGCAGAA
CCGGATGAATTCTCGATATCTTCAGCGAATTCAACATCGCTGTGCTCGCTGACGACCTC
GCTCAGGAATCCCGCCAGTTCCGTACAGACGTACCGTCCGGCATCGATCCCCCTCGAACAG
CTCGCTCAGCAGTGGCAGGACTTCGATGGCTGCCCCGCTCGCTTTGAACGAAGACAAACCG
CGTGGCCAGATGCTCATCGACATGACTAAGAAATACAATGCTGACGCCGTCGTCATCTGC
ATGATGCGTTTCTGCGATCCTGAAGAATTCGACTATCCGATTTACAAACCGGAATTTGAA
GCTGCTGGCGTTTCGTTACACGGTCCTCGACCTCGACATCGAATCTCCGTCCCTCGAACAG
CTCCGCACCCGTATCCAGGCTTTCTCGGAAATCCTCTAA (SEQ ID NO:25)

Figure 19

MSQIDELISKLOEVSNHPQKTVLNYKKQGKGLVGMPYYAPEEIVYAAGYLPVGMFGSQN
PQISAARTYLPFFACSLMQADMELQLNGTYDCLDAVIFSVPCDTLRCMSQKWHGKAPVIV
FTQPQNRKIRPAVDFLKAEYEHVTELGRILNVKISDLAIQEAIKVYNENRQVMREFCDV
AAQYPQIFTPIKRHDVIKARWFMDKAEHTALVRELIDAVKKEPVQPWNGKKVILSGIMAE
PDEFLDIFSEFNIAVVADDLAQESRQFRTDVPSGIDPLEQLAQWQDFDGCPLALNEDKP
RGQMLIDMTKKYNADAVVICMMRFGDPEEFDYPIYKPEFEAAGVRYTVLDLDIESPSLEQ
LRTRIQAQFSEIL (SEQ ID NO:26)

Figure 20

SEQ ID NO:25	1 atgagtcagatcgacgaacttatcagcaaattacaggaagtatccaacca
SEQ ID NO:27	1 atggct---atcagtgcaacttattgaagagttccaaaaagtat-ctgccca
SEQ ID NO:28	1 -----atgatgaaattaaaggcaattgaaaagttgatgcaaaaat
SEQ ID NO:29	1 atgtcaacttgtcaccgatctaccgccattttcgatcagttctctgaagc
SEQ ID NO:25	51 tccccagaag-----ac-----ggttttg---aattataaaaaa
SEQ ID NO:27	47 gccc--gaag-----ac-----catgctggccaaatataaaagcc
SEQ ID NO:28	41 tcgccagtag-----aaaagaacagctatat---aagcaaaaagaa
SEQ ID NO:29	51 tcgccagacaggctttctcac-----cgtcattg---gatctcaaggag
SEQ ID NO:25	82 cagggtaaaggcctcgtaggca--tgatgcactactacgctccggaagaa
SEQ ID NO:27	79 cagggcaaaaaagccatcggt--gcctgccgtactatgttccggaagaa
SEQ ID NO:28	79 gaaggtagaaaagtttttgaa--tgttctgtgcctatgttccaatagaa
SEQ ID NO:29	91 cgcggcattccgctggttggcacttactgcacctttatgc--cgcaagag
SEQ ID NO:25	130 atcgatatgctgcaggctacctcccggtaggcatgt---tcggttccca
SEQ ID NO:27	127 ctggtctatgctgcaggcatggttcccatgggtgtat---ggggtgcaa
SEQ ID NO:28	127 ataattttagcagcaaatgcaatcccgattggtttgt---gtggaggtaa
SEQ ID NO:29	139 atcccgatggcagccgg-----tgcggttggtttcgctctgttccac
SEQ ID NO:25	177 -----gaacccgcag-atctccgcagctcgtacgtaccttcccggtt
SEQ ID NO:27	174 -----tgccaaacaggaagtcggttccaaggaa-tactgtgcttccctt
SEQ ID NO:28	174 -----aaatgacaca-atcccaatagcagaggaggatttgccaagaaa
SEQ ID NO:29	183 ctctgatgaaacc-----attgaagaagcggagaaaagatctgccgcgcaa
SEQ ID NO:25	219 cgcttgctccttgatgcaggctgacatggaactccagctcaacggca---
SEQ ID NO:27	216 ctactgcaccattgcccagcagctctctggaaatgctgctggacggga---
SEQ ID NO:28	216 cctatgcccattaataaaatcatcctatggttttaag---aaggca---
SEQ ID NO:29	228 cctctgcccgtga-----ttaaagcagctacggct--tcggcaaaa
SEQ ID NO:25	266 cctatgactgcctcgacgctgttatcttctcc---gttcc-tgcy---
SEQ ID NO:27	263 ccctggatgggttggacgggatcatca-ctcc---ggtactgtgtg---
SEQ ID NO:28	259 ---aaacctgccttactttg-aagcatctgatatagttatt-ggag---
SEQ ID NO:29	269 ccgataaatgcccctac---ttctacttttc---ggatct-ggtggtc
SEQ ID NO:25	308 ----acactctccgctgcatgagccagaaat-----gg-----c-
SEQ ID NO:27	305 ----atacctgctgccatgagccagaaacttcaaagtgg-----cc
SEQ ID NO:28	302 ----aaact-----acctgtgaa-----gg-----a-
SEQ ID NO:29	310 ggtgaaaccacctgacgagcggcaaaaagaaaa-----tgtatgaatac-
SEQ ID NO:25	338 ----acggcaaaagct----ccggtcatcg-tcttcacacagccgcagaac
SEQ ID NO:27	343 atgaaagacaagatg----ccggttattt-tcctggctcatccccaggctc
SEQ ID NO:28	319 ----aagaagaagat----gtttgagttgatggagagattggtgccaatg
SEQ ID NO:29	352 ----atggcggagtttaagcctgttcatg-tgatgcaattgcccacagc
SEQ ID NO:25	379 cgtaaga-tccgcccggc-----tgctgatttccctcaaag-ct
SEQ ID NO:27	388 cgtcagaatgcgcggc-----aagc-agttcacctatg-at
SEQ ID NO:28	361 catataa-tgcacctccacacatgaaagatgaagattctttgaaaatct
SEQ ID NO:29	397 gttaagg-acgatgcctc-----gcgtgcgttatggaaag-cc
SEQ ID NO:25	415 gaat--acgaacatgtc---cgt-----acgg--aattgg----gacg
SEQ ID NO:27	424 gcct--acagcgaagt-----ga-----aaggccatctgg----aaga
SEQ ID NO:28	410 ggattaaagaagttgaaaagcta-----aaag--aattggttgagaaa
SEQ ID NO:29	433 ga-----gatgctgcg---cttgcaaaaacgg--tagaag----aacg

SEQ ID NO:25	447	tatcctcaacgtaaaa--atctccgacctggctatccaggaagctatcaa
SEQ ID NO:27	456	aatctgcggccatgaa--atcaccaatgatgccatcctggatgccatcaa
SEQ ID NO:28	451	gagactggaaataaaataacagaggaaggttaaagagacagtgtgataa
SEQ ID NO:29	468	ttttgggcacg---ag--attagcgaagatgctctgcgcgatgccattgc
SEQ ID NO:25	495	agtatataacgaaaaccgtcaggttatgctggaattct-----gcg
SEQ ID NO:27	504	agtgtacaacaagagccgtgctgccgcgcgaattct-----gca
SEQ ID NO:28	501	agtaaataaagtta---gggagttgtttataaaactct-----atg
SEQ ID NO:29	513	gctgaaaaaccgcgaacgtcgcgcactggctaatttttatcatcttgggc
SEQ ID NO:25	536	acgtagctgctcag-----taccgcgagatcttcaactccgataaa--acg
SEQ ID NO:27	545	aactggc--caacg-----aacatcctgatctgatccgggcttccgtacg
SEQ ID NO:28	539	a-attgaggaagaa-----taaac-cag-----ctccaattaa---ggg
SEQ ID NO:29	563	agttaaatcctccggcgcttagcggcag--cgacattctgaaagt---ggt
SEQ ID NO:25	579	tcattgacgtcatc-----aaag---cccgttg-----ttca
SEQ ID NO:27	588	ggccaccgtactg-----cgtg---ccgcttac-----ttca
SEQ ID NO:28	573	tttagatgtttta-----aaattattccagtttgcttatttat
SEQ ID NO:29	609	ttacggcgcaaccttccggttcgataaaag---aggcgttg-----atca
SEQ ID NO:25	608	tggacaaagctgaacacaccgcttttggtccgcgaactcatcgacgctgtc
SEQ ID NO:27	617	tgctgaaggatgaatacacccgaaaagctggaagaactgaacaagg-----
SEQ ID NO:28	611	tggtattgatgacacaatagggtatttagaggatttaattgaggagtta
SEQ ID NO:29	650	atgaactggatgcaatgaccgc-----ccgcg--ttcgtcagcagtgagg
SEQ ID NO:25	658	aagaa-----ag--aaccggtacagccgtggaat-----ggcaaaaaa
SEQ ID NO:27	662	aactg-----gc--agctgctcctgccggcaagttcagcggccacaaa
SEQ ID NO:28	661	gaggagagagttaa---aaaaggagaaggttatgaa-----ggaaagaga
SEQ ID NO:29	692	aagaa-----ggccagcgactggaccgcgctccg-----cgcatttta
SEQ ID NO:25	694	gtcatcctctccggt-----atcatggcagaaccggatgaattcct---
SEQ ID NO:27	703	gtggttggttccggc-----atcatctacaacacgcccggcatcct---
SEQ ID NO:28	703	attttaataactggtgtgccaatgggtgctggaaacaataagattgt---
SEQ ID NO:29	730	atcaccggctgcccg-----attggcggcgacgcagaaaaagtggtgcg
SEQ ID NO:25	735	cgatatcttcagcgaatt-caacatcgctgtcgtcgtgacgacctc-gc
SEQ ID NO:27	744	gaaagccatggatgacaa-caaactggccattgctgctgatgactgc-gc
SEQ ID NO:28	750	tgaattattgaggaagt-tggaggagtagttgttggtgaaagaagctgc
SEQ ID NO:29	774	cgcgat-tgaagagaatggcggctgggttgcggttatgaaaactgc-ac
SEQ ID NO:25	783	tcagga-atccccgccagttccgtacagacgtaccgtccggcatcgatccc
SEQ ID NO:27	792	ttatga-aagccgcagctttgccgtggatgctccggaagatctgga---c
SEQ ID NO:28	799	actgga-a-----caagattctttgaaaactttgttgagg--gctatagc
SEQ ID NO:29	822	cggggcgaaaagcgaccgagcaatgc-gtggcagaaaacggg---cgatgtc
SEQ ID NO:25	832	ctcgaacagctcgctcag-----cagtg-----caggacttcgat-g
SEQ ID NO:27	838	aacggactgcatgctctggctgtacagttctccaaacagaagaacgat-g
SEQ ID NO:28	841	gtagaggacattgcaaa-----aaga-tacttt-a
SEQ ID NO:29	868	tacgacgcgctggcggat-----aaatat-----ctgg---cgattg
SEQ ID NO:25	869	---gctgcccgctcgctttgaa-----cgaagacaaaaccgcg-tggccag
SEQ ID NO:27	887	ttctgctgtacgatcc---tgaatttgccaagaataccggttctgaaacac
SEQ ID NO:28	869	---aaatcccatgtgcttgta-----gatttaaaaacgat-gagagag
SEQ ID NO:29	902	---gctgtc-ctgtgtttcgc-----cga--acgatcagcg-cctgaaa
SEQ ID NO:25	910	atgctcatcgaca-----tgactaagaaatacaatgctgacgccgtcgtc
SEQ ID NO:27	934	gttggca---atc-----tggtaaaagaagcggcgagaggactgac
SEQ ID NO:28	908	ttgaaaataaagagattgggttaaagagttggacgtcgatggagttgtt
SEQ ID NO:29	940	atgctcagccaga-----tggtggaggaaatcacggtcgatggcgtagtt

SEQ ID NO:25	955	atctgcatgatgcggttctgcgacccgaagaattcgactatc---cgat
SEQ ID NO:27	976	gtgttcatgatgcagttctgcgacccgaagaatggaatatc---ctga
SEQ ID NO:28	958	tattacactttgcagttatgccatacatttaacatagagggag---ctaa
SEQ ID NO:29	985	gatgtgattttgcaggcgtgccatacctacgcggtggaatcgctggcgat
SEQ ID NO:25	1002	ttacaaaccggaatttgaagctgctgg---cgttcgttacacggtcctc
SEQ ID NO:27	1023	tctgaagaaggctctggatgccacca---cattcctcatgtgaagatt
SEQ ID NO:28	1005	ggtagaggaggcattaaaagaggagg---cattc---caattata
SEQ ID NO:29	1035	t---aaacgtcatgtgcgcagcagcaccaacattccttatatcgctatt
SEQ ID NO:25	1048	gacctcgacatcgaatctccgtccctcgaa-----cagctccgcacccg
SEQ ID NO:27	1069	ggtgtggaccagatgacccgggactttggt-----caggcccagaccgc
SEQ ID NO:28	1045	agaattgaaactgactattctgaaagtgatagagagcagttaaaaacaag
SEQ ID NO:29	1081	gaaacagactactccacctcggtgtcggtg---cagctcagtagcccg
SEQ ID NO:25	1092	tatccaggctttctcggaatcctctaa
SEQ ID NO:27	1113	tctggaagctttcgagaaagcctgtaa
SEQ ID NO:28	1095	gttgaggcattttattgagatgatttaa
SEQ ID NO:29	1125	tgtcgcgccctttattgagatgctgtaa

Figure 21

SEQ ID NO:26	1 msqidelisklqevsnhpqk---tvlnykkqgkglvgmmpypyapeei vya
SEQ ID NO:30	1 -maisalieefqkvsaspkt---mlakykaqgkkaigclpyyvpeelvya
SEQ ID NO:31	1 mmkl-kaiekimqkf asrke---qlykqkeegrkvfgmfca yvpieilila
SEQ ID NO:32	1 mslvtdlpaifdqfsearqtgfltvmdlkergiplvgtyctfmpqeipma
SEQ ID NO:26	48 agylpvgmfgsqnpqisaartylppfacslmqadmelqlngt---ydc--
SEQ ID NO:30	47 agmvpmgvwcngkqevrskeycasfyctiaqqsilemldgt---ldg--
SEQ ID NO:31	47 anaipvglcggkndtipiaeedlprnlcplikssygfkkaktcp yfea--
SEQ ID NO:32	51 agavvslcstsdetieeaekdlprnlcplikss---ygfgkt---dkc py
SEQ ID NO:26	93 ---ldavifsvpcdtlrcmsqkwh----gkapvivftqpqnkrirpavdf
SEQ ID NO:30	92 ---ldgiitpvlcdtlrpnasqnfkvamkdkmpviflahpqvrqnaagkqf
SEQ ID NO:31	95 ---sdivigettceggkkmfelme----rlvpmhimhlp-hmkdedslki
SEQ ID NO:32	96 fyfsdlvvgettcdgkkmeyema----efkpvhvmqlpnsvkddasral
SEQ ID NO:26	136 lkaeyehvrtelgrilnvkisdlaiqeai kvynenrqvmrefcdva aqyp
SEQ ID NO:30	139 tydaysevkgheleicgheitndaildaikvynksraarrefcklanehp
SEQ ID NO:31	137 wikeveklkelveketgnkiteeklketvdkvnkvrelfyklyelrknkp
SEQ ID NO:32	142 wkaemlrqlktveerfghesedairdaialknrreralanfyhlgqlnp
SEQ ID NO:26	186 qiftpikrhdivk----arwf---mdkaehtalvrelidavkk--epvqp
SEQ ID NO:30	189 dlipasvratvlr----aayf---mlkdeytekleelnkelaa--apagk
SEQ ID NO:31	187 ---apikgldvlk---lfqfaylldiddttigiledlieeleervkkg eg
SEQ ID NO:32	192 ---palsgsdilkvvygatfr---fdk---ealinel-damta--rvrq q
SEQ ID NO:26	227 wn-gkk-----vilsg--imaepdefldifsefniavvaddlaqesrqf
SEQ ID NO:30	230 fd-ghk-----vvvsg--iiyntpgilkamddnklai aaddcayesrsf
SEQ ID NO:31	230 ye-gkr-----ilitgcpmvagnnkiveiieevggvvvgeesctgtrff
SEQ ID NO:32	230 weegqzldprprilitgcpiggaaekvvraieenggvvvyenctgakat
SEQ ID NO:26	268 rtdvpsgidp-leqlaqwqdfdgcp lained---kprgqmlidmtkkyn
SEQ ID NO:30	271 avdapedldnglhalavqfskqkndvillydpefakntrsehvgnlvkesg
SEQ ID NO:31	273 enfv-egys--vediakryfkip-cacr fkn---e-rvenikrlvkeld
SEQ ID NO:32	280 eqcvaetgdv-ydalackylai-gcscvspnd---q-rlkmlsqmveeyq
SEQ ID NO:26	314 adavvicmmrfcdpeefdyipykpef-eaagvrytvldldiespsleqlr
SEQ ID NO:30	321 aeglivfmmqfcdpeemeypdlkkal-dahhiphvkigvdqmrtdfggaq
SEQ ID NO:31	315 vdgvvyytlqychtfniegakveeal-keegipli rietdysesdreqlk
SEQ ID NO:32	324 vdgvvdvilqachtyaveslaikrhvrq qhnpylaiaetdysts dvgqls
SEQ ID NO:26	363 triqafseil
SEQ ID NO:30	370 taleafaesl
SEQ ID NO:31	364 trleafiemi
SEQ ID NO:32	374 trvaafiemi

Figure 22

1	CGACGGGCCG	GGCTGGTATC	ATTCTAGTCA	GTAATTCACC	TTTGAAAAAT	TTTCACAAAG
61	GCAGTACGAC	AGAAGCGTCG	ATACATTCCA	TTTAGCAGGA	GGAAGTTACG	GTAATGAGAA
121	AAGTAGAAAT	CATTACAGCT	GAACAAGCAG	CTCAGCTCGT	AAAAGACAAC	GACACGATTA
181	CGTCTATCGG	CTTGTGCAGC	AGCGCCCATC	CGGAAGCACT	GACCAAAGCT	TTGGAAAAAC
241	GGTTCTTGGA	CACGAACACC	CCGCAGAACT	TGACCTACAT	CTATGCAGGC	TCTCAGGGCA
301	AACGCGATGG	CCGTGCCGCT	GAACATCTGG	CACACACAGG	CTTTTGA AAA	CGCGCCATCA
361	TCGGTCACTG	GCAGACTGTA	CCGGCTATCG	GTAAACTGGC	TGTCGAAAAC	AAGATTGAAG
421	CTTACAACTT	CTCGCAGGGC	ACGTTGGTCC	ACTGGTTCCG	CGCCTTGGCA	GGTCATAAGC
481	TCGGCGTCTT	CACCGACATC	GGTCTGGAAA	CTTTCCTCGA	TCCCCGTCAG	CTCGGCGGCA
541	AGCTCAATGA	CGTAACCAAA	GAAGACCTCG	TCAAACGTAT	CGAAGTCGAT	GGTCATGAAC
601	AGCTTTTCTA	CCCGACCTTC	CCGGTCAACG	TAGCTTTCTT	CCGCGGTACG	TATGCTGATG
661	AATCCGGCAA	TATCACCATG	GACGAAGAAA	TCGGGCCTTT	CGAAAGCACT	TCCGTAGCCC
721	AGGCCGTTCA	CAACTGTGGC	GGTAAAGTTC	TCGTCCAGGT	CAAGAGCGTC	GTGCTCAGC
781	GCAGCTCGA	CCCGCGCATG	GTCGAAGTCC	CTGGCATCTA	TGTCGACTAC	GTGCTCGTAG
841	CAGCTCCGGA	AGACCATCAG	CAGACGTATG	ACTGCGAATA	CGATCCGTCC	CTCAGCGGTG
901	AACATCGTGC	TCCTGAAGGC	GCTAGCGATG	CAGCTCTCCC	CATGAGCGCT	AAGAAAATCA
961	TCGGCCGCCG	CGCGCCTTTG	GAATTGACTG	AAAACGCTGT	CGTCAACCTC	GGCGTCGGTG
1021	CTCCGGAATA	CGTTGCTTCT	GTTGCCGGTG	AAGAAGGTAT	CGCCGATACC	ATTACCCTGA
1081	CCGTCGAAGG	TGGCGCCATC	GGTGGCGTAC	GGCAGGGCGG	TGGCCGCTTC	GGTTCGTGCC
1141	GCAATGCCGA	TGCCATCATC	GACCACACCT	ATCAGTTCGA	CTTCTACGAT	GGCGCGGCTC
1201	TGGACATCGC	TTACCTCGGC	CTGGCCAGT	GCGATGGCTC	GGGCAACATC	AACGTCAGCA
1261	AGTTCGGTAC	TAACGTTGCC	GGCTGGGGCG	GTTTCCCCAA	CATTTCCAG	CAGACACCGA
1321	ATGTTTACTT	CTGGGCGACC	TTACGCGCTG	GCGGCTTGAA	AATCGCTGTC	GAAGACGGCA
1381	AAGTCAAGAT	CCTCCAGGAA	GGCAAAGCCA	AGAAGTTTAT	CAAAGCTGTC	GACCAGATCA
1441	CTTTCAACGG	TTCTTATGCA	GCCCGCAACG	GCAACACAGT	TCTCTACATC	ACAGAACGCT
1501	GCGTATTGGA	ACTGACCAAA	GAAGGCTTGA	AACTCATCGA	AGTCGCACCG	GGCATCGATA
1561	TTGAAAAGA	TATCCTCGCT	CACATGGACT	TCAAGCCGAT	CATTGATAAT	CCGAAACTCA
1621	TGGATGCCCG	CCTCTTCCAG	GACGGTCCCA	TGGGACTGAA	AAAATAAATC	TCTGCTGTAA
1681	AGGAGACTTT	ACTATGAAAC	CAATGAGACT	ACATCAGCTA	GGCATTTGTC	TGCGAGCCTT
1741	AGAAAAAGCC	CATGAATTCA	TGCAGAAATA	TGGACTTGAA	ATCGACTATG	CCGGCTATGT
1801	CGATGCTTAC	CAGGCTGATC	TCATTTTAC	TAAGTTTGGT	GAATTTGCCA	GCCCGATTGA
1861	AATGATTATC	CCGCACTCCG	GTGTGCTTAC	CCAATTCAAT	GGTGGCCGCG	GCGGCATTGC
1921	CCACATCGCC	TTCCGAAGTG	ACGATGTGCA	AGCTGTCCCG	CAGGAAATGG	AAGCAGATTG
1981	TCCGGGATGC	ATGTTAGAAA	AGAAAGCTGT	CCAGGGTACG	GACGACATTA	TCGTCAACTT
2041	CCGCCGCCCG	ACAACCAACC	AGGGTATCCT	CGTTGAATAT	GTTTCAGACG	CAGCACCTAT
2101	CACCGGCCGC	GGCGAAAAATC	CTTTCGTTAA	GAATCTCGGC	CCGGGAAAAG	GGAAGCTCAA
2161	CGAAACATGG	CATCCCATGC	GCCTGCACCA	TATCGGCATC	GTCTTGCCGA	CCTTGAAAAA
2221	GGCCCATGAA	TTCATCAAGA	CCAATGGTCT	GGAAGTGGAT	TATTCCGTTT	TCGTGCAAGC
2281	CTACCATCGC	GATCTCATTT	TCACTAAAAA	AGGTGAAAAC	AGTACGCCTA	TGGAATTCAT
2341	TATTCCCGGT	GAAGGGGTCC	TCAAAGATTT	CAATCATGGC	AGGGGAGGTA	TCGCTCATAT
2401	CGCCTTTGAA	GTGGATGATG	TCGAAAAGGT	ACGTGAGATT	ATGGAAGGCC	AGAAGCCTGG
2461	TTGCATGCTC	GAAAAGAAAG	CCGTCCGGGG	AACGGACGAT	ATCATCGTCA	ACTTCCGCGG
2521	TCCCAGCAGC	GACGCCGGCA	TCCTCGTCTA	ATATGTCCAG	ACCGTAGCTC	CCATCAATCG
2581	CAGCAATCCC	AACCCCTTTA	ATGATTGATT	TTTTATAAAG	AAAGGTGAAA	ACTGTGTATA
2641	CTCTCGGAAT	CGACGTTGGT	TCTTCTTCTT	CCAAGGCAGT	CATCCTGGAA	GATGGCAAGA
2701	AGATCGTCGC	CCATGCCGTC	GTTGAAATCG	GCACCGGTTT	GACCGGTCCG	GAACGCGTCC
2761	TGGACGAAAGT	CTTCAAAGAT	ACCAACTTAA	AAATTGAAGA	CATGGCGAAC	ATCATCGCCA
2821	CAGGCTATGG	CCGTTTCAAT	GTCGACTGCG	CCAAAGGCGA	AGTCAGCGAA	ATCACGTGCC
2881	ATGCCAAAGG	GGCCCTCTTT	GAATGCCCGG	GTACGACGAC	CATCCTCGAT	ATCGGCGGTC
2941	AGGACGTCAA	GTCCATCAAA	TTGAATGGCC	AGGGCCTGGT	CATGCAGTTT	GCCATGAACG
3001	ACAAATGCGC	CGCTGGTACG	GGCCGTTTCC	TCGACGTCAT	GTCGAAGGTA	CTGGAAATCC
3061	CCATGCTCTGA	AATGGGGGAC	TGGTACTTCA	AATCGAAGCA	TCCCGCTGCC	GTGACGAGTA
3121	CCTGCACGGT	TTTTGCTGAA	TCGGAAGTCA	TTTCCCTTCT	TTCCAAGAAAT	GTCCCGAAAG
3181	AAGATATCGT	AGCCGGTGTC	CATCAGTCCA	TCGCCGCCAA	AGCCTGCGCT	CTCGTGCGCC
3241	GCGTCGGTGT	CGGTGAAGAC	CTGACCATGA	CCGGCGGTGG	CTCCGCGCAT	CCCGGCGTCC
3301	TCGATGCGGT	ATCGAAAGAA	TTAGGTATTCT	CTGTCAGAGT	CGCTCTGCAT	CCCAAGCGCG
3361	TGGGTGCTCT	CGGAGCTGCT	TTGATTGCTT	ATGATAAAAT	CAAGAAATAA	GTCAAAGGAG

3421 AGAACAAAAT CATGAGTGAA GAAAAACAG TAGATATTGA AAGCATGAGC TCCAAGGAAG
3481 CCCTTGGTTA CTTCTTGCCG AAAGTGGATG AAGACGCACG TAAAGCGAAA AAAGAAGGCC
3541 GCCTCGTTTG CTGGTCCGCT TCTGTCGCTC CTCGGGAATT CTGCACGGCT ATGGACATCG
3601 CCATCGTCTA TCCGAAACT CACGCAGCTG GTATCGGTGC CCGTCACGGT GCTCCGGCCA
3661 TGCTCGAAGT TGCTGAAAAC AAAGGTTACA ACCAGGACAT CTGTTCTTAC TGCCGCGTCA
3721 ACATGGGCTA CATGGAACCT CTCAAACAGC AGGCTCTGAC AGGCGAAACG CCGGAAGTCC
3781 TCAAAAACCT CCCGGCTTCT CCGATTCCCT TCCGGATGT TGTCCTCACT TGCAACAACA
3841 TCTGCAATAC CTTGCTCAAA TGGTATGAAA ACTTGGCTAA AGAATTGAAC GTACCTCTCA
3901 TCAACATCGA CGTACCGTTC AACCATGAAT TCCCTGTTAC GAAACACGCT AAACAGTACA
3961 TCGTCGGCGA ATTCAAACAT GCTATCAAAC AGCTCGAAGA CCTTTGOGGC CGTCCCTTCG
4021 ACTATGACAA ATTCTTCGAA GTACAGAAAC AGACACAGCG CTCCATCGCT GCCTGGAACA
4081 AAATCGCTAC GTACTTCCAG TACAAACCGT CGCCGCTCAA CGGCTTCGAC CTCTTCAACT
4141 ACATGGGCCT CGCCGTTGCT GCCCGCTCCT TGAACACTC GGAAATCAGC TTCAACAAAT
4201 TCCTCAAAGA ATTGGACGAA AAAGTAGCTA ATAAGAAATG GGCTTTCCGT GAAAACGAAA
4261 AATCCCGTGT TACTTGGGAA GGTATCGCTG TCTGGATCGC TCTCGGCCAC ACCTTCAAAG
4321 AACTCAAAGG TCAGGGCGCT CTCATGACTG GTTCCGCTTA TCTGGCATG TGGGACGTTT
4381 CCTACGAACC GGGCGACCTC GAATCCATGG CAGAAGCTTA TCCCGTACA TACATCAACT
4441 GCTGCCTCGA ACAGCGCGGT GCTGTTCTTG AAAAAGTTGT CCGCGATGGC AAATGCGACG
4501 GCTTGATCAT GCACCAGAAC CGTTCCTGCA AGAACATGAG CCTCCTCAAC AACGAAGGCG
4561 GCCAGCGCAT CCAGAAGAAC CTCGGCGTAC CGTACGTCAT CTTGACGGC GACCAGACCG
4621 ATGCTCGTAA CTCTCGGAA GCACAGTTGG ATACCCGCGT AGAAGCTTTG GCAGAAATGA
4681 TGGCAGACAA AAAAGCCAAT GAAGGAGGAA ACCACTAATG AGTCAGATCG ACGAACTTAT
4741 CAGCAAATTA CAGGAAGTAT CCAACCATCC CCAGAAGAGC GTTTTGAATT ATAAAAACA
4801 GGGTAAAGGC CTCGTAGGCA TGATGCCCTA CTACGCTCCG CAGAGAAATCG TATATGCTGC
4861 AGGCTACCTC CCGGTAGGCA TGTTCCGTTT CCAGAACCG CAGATCTCCG CAGCTCGTAC
4921 GTACCTTCCT CCGTTCGCTT GCTCCTTGAT GCAGGCTGAC ATGGAACCTC AGCTCAACCG
4981 CACCTATGAC TGCTCGACG CTGTTATCTT CTCGCTTCTT TGCGACACTC TCGCTGTCAT
5041 GAGCCAGAAA TGGCAGGGCA AAGCTCCGCT CATCGTCTTC ACACAGCCCG AGAACCCTAA
5101 GATCCGCCCG GCTGTCGATT TCCTCAAAGC TGAATACGAA CATGTCCGTA CGGAATTGGG
5161 ACGTATCCTC AACGTAAAAA TCTCCGACCT GGCTATCCAG GAAGCTATCA AAGTATATAA
5221 CGAAAAACCGT CAGGTTATGC GTGAATTCTG CGACGTAGCT GCTCAGTACC CGCAGATCTT
5281 CACTCCGATA AAACGTCATG ACGTCATCAA AGCCCGCTGG TTCATGGACA AAGCTGAACA
5341 CACCGCTTTG GTCCGCGAAC TCATCGACGC TGTCAAGAAA GAACCGGTAC AGCCGTGGAA
5401 TGGCAAAAAA GTCATCCTCT CCGGTATCAT GGCAGAACCG GATGAATTCC TCGATATCTT
5461 CAGCGAATTC AACATCGCTG TCGTCGCTGA CGACCTCGCT CAGGAATCCC GCCAGTTCCG
5521 TACAGACGTA CCGTCCGGCA TCGATCCCCT CGAACAGCTC GCTCAGCAGT GGCAGGACTT
5581 CGATGGCTGC CCGCTCGCTT TGAACGAAGA CAAACCGCGT GGCCAGATGC TCATCGACAT
5641 GACTAAGAAA TACAATGCTG ACGCCGTGCT CATCTGCATG ATGCGTTTCT GCGATCCTGA
5701 AGAATTGAC TATCCGATTT ACAAAACCGA ATTTGAAGCT GCTGGCGTTC GTTACACGGT
5761 CCTCGACCTC GACATCGAAT CTCGCTCCCT CGAACAGCTC CGCACCCGTA TCCAGGCTTT
5821 CTCGGAATC CTCTAAGAA CTGCTGAATC ATCAAACATC TGGGCGGGAC TCCGAAAGGT
5881 GCCTGCTACA TGATACATTG CCTGTTTTCA GGCAGACAGA TTTGCAGCTT GCGGCCCCCA
5941 TTGTACGGGC TGCAAGCTGT CAATGATGCT TTAAAGACCG CTCTGCCGTT TTTAAATAAA
6001 AACATAAAAC CATATATAAT CTATTAGGAG GAAACTCAAT CATGGAATTC AAACCTTCTG
6061 AATTACAGCA AGATATCGCA AATCTCGCAA AAGATTTGCG AGAAAAAAA TTAGCTCCCA
6121 CTGTCAAAGA GCGTGACGAA AAAGAAGTTT TCGATCGTGC TATCCTTGAC GAAGTGGGTA
6181 CTCTCGGCCT TCTCGGTATT CCCTGGGAAG AAGAAAACCG CGGCGTAGGC GCTGACTTCC
6241 TCAGCCTCGC AGTTGCTTGC GAAGAAGTAG CTAAAGTTAC CAGCCCGGGC CGTCTG (SEQ
ID NO:33)

Figure 23

ATGAAACCAATGAGACTACATCAGTAGGCATTGTCCTGCCGACCTTAGAAAAAGCCCAT
GAATTCATGCAGAATAATGGACTTGAAATCGACTATGCCGGCTATGTCGATGCTTACCAG
GCTGATCTCATTTTCACTAAGTTTGGTGAATTTGCCAGCCCGATTGAAATGATTATCCCG
CACTCCGGTGTGCTTACCCAATTCAATGGTGGCCGCGCGGCATTGCCACATCGCCTTC
GAAGTGGACGATGTCGAAGCTGTCCGCCAGGAAATGGAAGCAGATTGTCCGGGATGCATG
TTAGAAAAGAAAGCTGTCCAGGGTACGGACGACATTATCGTCAACTTCGCGCGCCCGACA
ACCAACCAGGGTATCCTCGTTGAATATGTTTCAGACGACAGCACCTATCAGGGGCCGCGGC
GAAATCCTTTCGTTAAGAATCTCGGCCCGGAAAAAGGGAAGCTCAACGAAACATGGCAT
CCCATGCGCCTGCACCATATCGGCATCGTCTTGCCGACCTTGAAAAAGGCCCATGAATTC
ATCAAGACCAATGGTCTGGAAGTGGATTATTCCGGTTTCGTCGACGCCTACCATGCGGAT
CTCATTTTCACTAAAAAGGTGAAAACAGTACGCCTATCGAATTCATTATCCCCGTGAA
GGGGTCCTCAAAGATTTCAATCATGGCAGGGGAGGTATCGCTCATATCGCCTTTGAAGTG
GATGATGTCGAAAAGGTACGTCAGATTATGGAAAGCCAGAAGCCTGGTTGCATGCTCGAA
AAGAAAGCCGTCCGGGGAACGGACGATATCATCGTCAACTTCGCGCGTCCAGCACGGAC
GCCGGCATCCTCGTCGAATATGTCCAGACCGTAGCTCCCATCAATCGCAGCAATCCCAAC
CCTTTTAATGATTGA (SEQ ID NO:34)

Figure 24

MKPMRLHHVGIVLPTLEKAHEFMQNNGLEIDYAGYVDAYQADLIFTKFGEFASPIEMIIP
HSGVLTQFNNGRGGIAHIAFEVDDVEAVRQEMEADCPGCMLEKKAVQGTDDIIVNFRRPT
TNQGILVEYVQTTAPITGRGENPFVKNLGPEKGKLNETHWPMRLHHIGIVLPTLEKAHEF
IKTNGLVDYSGFVDAYHADLIFTKKGENSTPIEFIIIPREGVLKDFNHGRGGIAHIAFEV
DDVEKVRQIMESQKPGCMLEKKAVRGTDIIVNFRRPSTDAGILVEYVQTVAPINRSNP
PFND (SEQ ID NO:35)

Figure 25

ATGGAATTCAAACCTTCTGAATTACAGCAAGATATCGCAAATCTCGCAAAAGATTTCGCA
GAAAAAAATTAGCTGCCACTGTCAAAGAGCGTGACGAAAAAGAAGTTTTCGATCGTGCT
ATCCTTGACGAAGTGGGTACTCTCGGCCTTCTCGGTATCCCTGGGAAGAAGAAAACGGC
GGCGTAGGCGCTGACTTCCTCAGCCTCGCAGTTGCTTGCGAAGAAGTAGCTAAAGTTACC
AGCCCGGGCCGTCG (SEQ ID NO: 36)

Figure 26

MEFKLSELQQDIANLAKDFAEKKLAPTVMKERDEKEVFDRAILDEVGTLGLLGIPWEEENG
GVGADFLSLAVACEEVAKVTSPGR (SEQ ID NO:37)

Figure 27

```

1  GTGAGCACAC ACTTGATAGC TGATGCCGTC AATGATCAGT TGTTCGTCTA TAGCAGGCTG
61  AAAGGACATG GGTTTGGTCA CAGTCTGAGC AGTTGCAGGC AGTCAAACAC GTTCGTAAC
121 ACGCTGTAGA TGATATAAGC AGTATAACCAT CTTGCTACGC TCTCGTTGAT CAGGTTGAAT
181 GCTTTGAGGA AGGTCAAGCGC AATAGCCATG CCTCTTGTTT CCAGAACATG GCATGGGGAT
241 GGATCGACGG TACCTGTGCG GATGCATGCT ATGCGTGGCA TTCATATCAT CAACCAGAA
301 TTGATCTTGA ACTACACAGC AATTCTGCGC GTTATGCAAG TGTCTTCGGT CAGATGGTGA
361 ACAATTCTCA ATTGTTGAGG TCTTGACGAA TTGCGTTATA CACTGTAGGC TATAGTATGC
421 ACCCCTTGTT ATCTATATCA CAACCGGTCT ATTAGCATTG GCGTCAAGGA GGATGGTCTGA
481 TGATCGACAC TCGGCCCTT GCCCACCAC GGGGGCCCGG CTCTAATCCG ATTCGGGATC
541 GAGTTGATTG GGAAGCTCAG CGCGCTGCTG CGCTGGCAGA TCCCGGTGCC TTTCATGGCG
601 CGATTGCCCG GACAGTTATC CACTGGTACG ACCACAACA CCATTGCTGG ATTCGCTTCA
661 ACGAGTCTAG TCAGCGTTGG GAAGGGCTGG ATGCCGCTAC CGGTGCCCT GTAACGGTAG
721 ACTATCCCGC CGATTATCAG CCCTGGCAAC AGGCGTTTGA TGATAGTGAA CCGCCGTTTT
781 ACCGCTGGTT TAGTGGTGGG TTGACAAATG CCTGCTTAA TGAAGTAGAC CGGCATGTCA
841 TGATGGGCTA TGGCGACGAG GTGGCCTACT ACTTTGAAGG TGACCGCTGG GATAACTCGC
901 TCAACAATGG TCGTGGTGGT CCGGTTGTCC AGGAGACAAT CACGCGGGG CGCCTGTTGG
961 TGGAGGTGGT GAAGGCTGCG CAGGTGTTGC GTGATCTGGG CCTGAAGAAG GGTGATCGGA
1021 TTGCTCTGAA TATGCCGAAT ATTATGCCCG AGATTATTA TACGGAAGCG GCAAAACGAC
1081 TGGGTATTCT GTACACGCGG GTCTTGGGTG GCTTCTCGGA CAAGACTCTT TCCGACCGTA
1141 TTCACAATGC CCGTGACAGA GTGGTGATTA CCTCTGATGG TCGGTACCGC AACGCGCAGG
1201 TGGTGCCCTA CAAAGAAGCG TATACCGATC AGGCGCTCGA TAAGTATATT CCGGTTGAGA
1261 CGGCGCAGGC GATTGTTGCG CAGACCTGG CCACCTTGGC CCGTACTGAG TCGCAGCGCC
1321 AGACGATCAT CACCGAAGTG GAGGCCGAC TGGCCGGTGA GATTACGGTT GAGCGCTCGG
1381 ACGTGATGCG TGGGGTTGGT TCTGCCCTCG CAAAGCTCCG CGATCTTGAT GCAAGCGTGC
1441 AGGCAAAGGT GCGTACAGTA CTGGCGCAGG CGCTGGTGA GTCCGCCCG CGGGTTGAAG
1501 CTGTGGTGGT TGTGGCTCAT ACCGGTACG AGATTTTGTG GAACGAGGGG CGAGATCGCT
1561 GGAGTCACGA CTTGCTGGAT GCTGCGCTGG CGAAGATTCT GGCCAATGCG CGTGCTGCCG
1621 GCTTTGATGT GCACAGTGAG AATGATCTGC TCAATCTCCC CGATGACCAG CTTATCCGTG
1681 CGCTCTACGC CAGTATTCCC TGTGAACCGG TTGATGCTGA ATATCCGATG TTTATCATTT
1741 ACACATCGGG TAGCACCGGT AAGCCCAAGG GTGTGATCCA CGTTCACGGC GGTATGTCG
1801 CCGGTGTGGT GCACACCTTG CCGGTCAAGT TTGACGCGA GCGGGTGAT ACGATATATG
1861 TGATCGCCGA TCCGGGCTGG ATCACCGGTC AGAGCTATAT GCTCACAGCC ACAATGGCCG
1921 GTCGGCTGAC CGGGGTGATT GCCGAGGGAT CACCGCTCTT CCCCTCAGCC GGGCGTTATG
1981 CCAGCATCAT CGAGCGCTAT GGGGTGCAGA TCTTTAAGGC GGGGTGTGAC TTCTCAAGA
2041 CAGTGATGTC CAATCCGAG AATGTTGAAG ATGTGCGACT CTATGATATG CACTCGCTGC
2101 GGGTTGCAAC CTTCTGCGCC GAGCCGGTCA GTCCGGGGT GCAGCAGTT GGTATGAGA
2161 TCATGACCCC GCAGTATATC AATCGTACT GGGCGAGCCA GCGCGGTGA ATTGTCTGGA
2221 CGCATTCTTA CCGTATCAG GACTTCCCGC TTCGTCCCGA TGCCCATACC TATCCCTTGC
2281 CCTGGGTGAT GGGTGATGTC TGGGTGGCG AAAGTATGA GAGCGGGACG ACGCGCTATC
2341 GGGTCGCTGA TTTGATGAG AAGGGCGAGA TTGTGATTAC CGCCCCGAT CCCTACCTGA
2401 CCGCACACT CTGGGGTGAT GTGCCGGTT TCGAGGCGTA CCTGCGCGGT GAGATTCCGC
2461 TGCGGGCCTG GAAGGGTGAT GCCGAGCGTT TCGTCAAGAC CTACTGGCGA CGTGGGCCAA
2521 ACGGTGAATG GGGCTATATC CAGGGTGATT TTGCCATCAA GTACCCCGAT GGTAGCTTCA
2581 CGCTCCACGG ACGCCCTGAC GATGTGATCA ATGTGTGGG CCACCGTATG GGCACCGAGG
2641 AGATTGAGGG TGCCATTTTG CGTGACCGCC AGATCAGGCC CGACTCGGCC GTCGGTAATT
2701 GTATTGTGGT CGGTGCGCG CACCGTGAGA AGGGTCTGAC CCGGGTTGCC TTCATTCAAC
2761 CTGCGCCTGG CCGTCATCTG ACCGGCGCG ACCGGCGCG TCTCGATGAG CTGGTGCGTA
2821 CCGAGAAGGG GCGGTCAGT GTCCAGAGG ATTACATCGA GGTCACTGCC TTTCCGAAA
2881 CCGCGAGCGG GAAGTATATG CGGCGCTTTT TCGCAATAT GATCTCGAT GAACCACTGG
2941 GTGATACGAC GACGTTGCGC AATCCTGAAG TGCTCGAAGA GATTGCAGCC AAGATCGCTG
3001 AGTGGAACG CCGTCAGCGT ATGGCCGAG AGCAGCAGAT CATCGAACG TATCGCTACT
3061 TCCGGATCGA GTATCACCCA CCAACGGCCA GTGCCGGTAA ACTCGCGGTA GTGACGGTGA
3121 CAAATCCGCC GGTGAACGCA CTGAATGAGC GTGCCCTCGA TGAGTTGAAC ACAATTGTTG
3181 ACCACCTGGC CCGTCGTCAG GATGTTGCG CAATTGTCTT CACCGGACAG GCGCCAGGA
3241 GTTTTGTGCG CGGCGCTGAT ATTCGCGAGT TGCTCGAAGA GATTACATCG GTTGAAGAGG
3301 CAATGGCCCT GCCGAATAAC GCCCATCTTG CTTTCCGCAA GATTGAGCGT ATGAATAAGC
3361 CGTGATCGC GCGGATCAAC GGTGTGGGCG TCGGTGGTGG TCTGGAATTC GCCATGGCCT

```

```

3421 GCCATTACCG GGTGCGGAT GTCTATGCCG AATTCGGTCA GCCAGAGATT AATCTGCGCT
3481 TGCTACCTGG TTATGGTGGC ACGCAGCGCT TGCCGCGCCT GTTGTACAAG CGCAACAACG
3541 GCACCGGTCT GCTCCGAGCG CTGGAGATGA TTCTGGGTGG GCGTAGCGTA CCGGCTGATG
3601 AGGCGCTGAA GCTGGGTCTG ATCGATGCCA TTGCTACCGG CGATCAGGAC TCACTGTGCG
3661 TGGCATGCGC GTTAGCCCGT GCCGCAATCG GCGCCGATGG TCAGTTGATC GAGTCGGGCTG
3721 CCGTGACCCA GGCTTTCCGC CATCGCCACG AGCAGCTTGA CGAGTGGCGC AAACCAGACC
3781 CCGGCTTTGC CGATGACGAA CTGCGCTCGA TTATCGCCCA TCCACGTATC GAGGGGATTA
3841 TCCGGCAGGC CCATACCGTT GGGCGCGATG CGCAGTGCA TCGGGCACTG GATGCAATCC
3901 GCTATGGCAT TATCCACGGC TTCGAGGCGG GTCTGGAGCA CGAGGCGAAG CTCTTTGCCG
3961 AGGCAGTGGT TGACCCGAAC GGTGGCAAGC GTGGTATTCT CGAGTTCTCT GACCGCCAGA
4021 GTGCGCCGTT GCCAACCCGC CGACCATTTG TTACACCTGA ACAGGAGCAA CTCTTGCGCG
4081 ATCAGAAAGA ACTGTTGCCG GTTGGTTTAC CCTTCTTCCC CGGTGTTGAC CGGATTCCGA
4141 AGTGGCAGTA GCGCGAGGCG GTTATTCTGT ATCCGGACAC CGGTGCGGCG -GCTCAGGGCG
4201 ATCCCATCGT GGCTGAAAAG CAGATTATTG TGCCGGTGGA ACGCCCCCGG GCCAATCAGG
4261 CGCTGATCTA TGTTCCTGGC TCGGAGGTGA ACTCAACGA TATCTGGGCG ATTACCGGTA
4321 TTCCGGTGTC ACGGTTTGAT GAGCACGACC GCGACTGGCA CGTTACGGT TCAGGTGGCA
4381 TCGGCTGAT CGTTGCGCTG GGTGAAGAGG CGCGACGCGA AGGCGGCTG AAGGTGGGTG
4441 ATCTGGTGGC GATCTACTCC GGCAGTCGG ATCTGCTCTC ACCGCTGATG GGCCTTGATC
4501 CGATGGCCGC -CGATTTCTG ATCCAGGGGA ACGACACGCG AGATGGATCG CATCAGCAAT
4561 TTATGCTGGC CCAGGCCCGG CAGTGTCTGC CCATCCCAAC CGATATGTCT ATCGAGGCAG
4621 CCGGCAGCTA CATCCTCAAT CTCGGTACGA TCTATCGCGC CCTCTTTACG ACGTTGCAAA
4681 TCAAGGCCGG ACGCACCATC TTTATCGAGG GTGCGGCGAC CGGTACGGT CTGGACGCAG
4741 CGCGCTCGGC GGCCCGGAAT GGTCTGCGCG TAATTGGAAT GGTCAGTTCT TCCTCACGTG
4801 CGTCTACGCT GCTGGCTGCG GGTGCCACG GTGCGATTAA CCGTAAAGAC CCGGAGGTTG
4861 CCGATTGTTT CACGCGCGTG CCCGAAGATC CATCAGCCTG GCGAGCCTGG GAAGCCGCGG
4921 GTCAGCCGTT GCTGGCGATG TTCCGGGCGC AGAACGACGG GCGACTGGCC GATTATGTGG
4981 TCTCGCACGC GGGCGAGACG GCCTTCCCGC GCAGTTTCCA GCTTCTCGGC GAGCCACGCG
5041 ATGGTCACAT TCCGACGCTC ACATTCTACG GTGCCACCAG TGGCTACCAC TTCACCTTCC
5101 TGGGTAAAGC AGGGTCAGCT TCGCCGACCG AGATGCTGCG GCGGGCCAAT CTCCGCGCCG
5161 GTGAGGCGGT GTTGATCTAC TACGGGGTTG GGAGCGATGA CCTGGTAGAT ACCGGCGGTC
5221 TGGAGGCTAT CGAGGCGCGG CGGCAAAATG GAGCGCGGAT CGTCGTCTGT ACCGTCAGCG
5281 ATGCGCAACG CGAGTTTGTC CTCTCGTTGG GCTTCGGGGC TGCCCTACGT GGTGTCGTCA
5341 GCCTGGCGGA ACTCAAACGG CGCTTCGGCG ATGAGTTTGA GTGGCCGCGC ACGATGCGGC
5401 CGTTGCCGAA CGCCCGCCAG GACCCGACGG GTCTGAAAGA GGCTGTCCGC CGCTTCAACG
5461 ATCTGGTCTT CAAGCCGCTA GGAAGCGCGG TCGGTGTCTT CTTGCGGAGT GCCGACAATC
5521 CGCGTGGCTA CCCGATCTG ATCATCGAGC GGGCTGCCCA CGATGCACTG GCGGTGAGCG
5581 CGATGCTGAT CAAGCCCTTC ACCGGACGGA TTGTCTACTT CGAGGACATT GGTGGGCGGC
5641 GTTACTCCTT CTTCGCACCG CAAATCTGGG TGCGCCAGCG CCGCATCTAC ATGCCGACGG
5701 CACAGATCTT TGGTACGCAC CTCTCAAATG CGTATGAAAT TCTGCGTCTG AATGATGAGA
5761 TCAGCGCCGG TCTGCTGACG ATTACCGAGC GGCAGTGGT GCGGTGGGAT GAACTACCCG
5821 AAGCACATCA GCGATGTGG GAAAATCGCC ACACGGCGGC CACTTATGTG GTGAATCATG
5881 CCTTACCACG TCTCGGCCTA AAGAACAGGG ACGAGCTGTA CGAGGCGTGG ACGGCCGGCG
5941 AGCGGTAGCG CGGATGGGTA TTGAACAGGT AACGGACGGA AGATCGAACC TTCCGTCCGT
6001 TATCTTTTGG CCGTCGAAGC GTGCTGAGCC GATTATCGTT GCGGTGGTTG TCCCGATGGG
6061 CAGACGCGCT CGAACCAGAT GATACCACCG ACGGCTATCG TCACCAAACC GGCGAAGACC
6121 AGGTAAGCCT CTGAAGGACG C (SEQ ID NO:38)

```

Figure 28

```

1  MIDTAPLAPP RAPRSNPIRD RVDWEAQRAA ALADPGAFHG AIARTVIHWY DPQHHCWIRF
61  NESSQRWEGL DAATGAPVTV DYPADYQPWQ QAFDDSEAPF YRWFSGGLTN ACFNEVDRHV
121 MMGYGDEVAY YFEGDRWDNS LNNRGGPPV QETITRRRL VEVVKAQVL RDLGLKKGDR
181 IALNMPNIMP QIYYTEAAKR LGILYTPVFG GFSDKTSLDR IHNAGARVVI TSDGAYRNAQ
241 VVPYKEAYTD QALDKYIPVE TAQAIVAQTL ATLPLTESQR QTIITEVEAA LAGEITVERS
301 DVMRGVGSAL AKLRDLDAV QAKVRTVLAQ ALVESPPRVE AVVVVRHTGQ EILWNEGRDR
361 WSHDLDAAL AKILANARAA GFDVHSENDL LNLDDQLIR ALYASIPCEP VDAEYPMFII
421 YTSGSTGKPK GVIHVHGGYV AGVVHTLRVS FDAEPGDTIY VIADEPGWITG QSYMLTATMA
481 GRLTGVIAGS SPLFPSAGRY ASIIERYGVQ IFKAGVTFLK TVMSNPQNV EIVRLYDMHSL
541 RVATFCAEPV SPAVQQFGMQ IMTPQYINSY WATEHGGIVW THFYGNQDFP LRPDAHTYPL
601 PWVMGDVWVA ETDESGTTRY RVADFDEKGE IVITAPYPYL TRTLWGDVPG FEAYLRGEIP
661 LRAWKGDAER FVKTYWRRGP NGEWGYIQGD FAIKYPDGSF TLHGRPDVVI NVSGHRMGTE
721 EIEGAILRDR QITPDSFVGN CIVVGAPHRE KGLTPVAFIQ PAPGRHLTGA DRRRLDELVR
781 TEKGAVSVPE DYIEVSAPFE TRSGKYMRRF LRNMMLDEPL GDTTLRNPE VLEEIAAKIA
841 EWKRRQMAE EQQIERYRY FRIEYHPPTA SAGKLAVTV TNPPVNALNE RALDELNTIV
901 DHLARRQDVA AIVFTGQGAR SFVAGADIRQ LLEEIHTVEE AMALPNNALH AFRKIERNMK
961 PCIAAINGVA LGGGLEFAMA CHYRVADVYA EFGQPEINLR LLPGYGGTQR LPRLLYKRNN
1021 GTGLLRALAM ILGGRSVPAD EALKLGLIDA IATGDQDLSL LACALARAAL GADGQLIESA
1081 AVTQAFRRH EQLDEWRKPD PRFADDELRS IIAHPRIERI IRQAHTVGRD AAVHRLDAI
1141 RYGIINGFEA GLEHEAKLFA EAVVDPNGGK RGIREFLDRQ SAPLPTRRPL ITPEQEQLLR
1201 DQKELLPVGS PFFPGVDRIK KWQYAAQAVR DDPOTGAAAHG DPIVAEKQII VPPERPRANQ
1261 ALIYVLASEV NFNDIWAITG IPVSRFDEHD RDWHVTGSGG IGLIVALGEE ARREGRLKVG
1321 DLVAIYSGQS DLLSPIMGLD PMAADFVIQG NDTPDGSHQQ FMLAQAPQCL PIPTDMSIEA
1381 AGSYILNLGT IYRALFTTLQ IKAGRTIFIE GAATGTGLDA ARSAARNGLR VIGMVSSSSR
1441 ASTLLAAGAH GAINRKDPEV ADCFTRVPED PSAAWAEAA GQPLLAMFRA QNDGRLADYV
1501 VSHAGETAFP RSFQLLGEPR DGHITLTFY GATSGYHFTF LGKPGSASPT EMLRRANLRA
1561 GEAVLIYYGV GSDDLVDVTDG LEAIEAARQM GARIVVTVS DAQREFVLSL GFGAALRGVV
1621 SLAELKRRFG DEFEPWRTMP PLPNARQDPQ GLKEAVRRFN DLVFKPLGSA VGVFLRSADN
1681 PRGYPDIIIE RAAHDALAVS AMLIKPFTGR IVYFEDIGGR RYSFFAPQIW VRQRRIYMPT
1741 AQIFGTHLSN AYEILRLNDE ISAGLLTITE PAVVPWDELP EAHQAMWENR HTAATYVNVH
1801 ALPRLGLKNR DELYEAWTAG ER (SEQ ID NO:39)

```


Figure 29

ATGAGTGAAGAGTCTCTGGTTCTCAGCACAAATTGAAGGGCCCATCGCCATCCTCACCCCTC
AATCGCCCCCAGGCCCTCAATGCGCTCAGTCCGGCCTTGATTGATGACCTCATTCGCCAT
TTAGAAGCCTGCGATGCCGATGACACAATCCGCGTGATCATTATCACCGGCGCGGACGG
GCATTTGCTGCCGGCGCTGATATCAAAGCGATGGCCAATGCCACGCCTATTGATATGCTC
ACCA GTGGCATGATTGCGCGCTGGGCACGCATCGCCGCGGTGCGCAAACCGGTGATTGCT
GCCGTGAATGGGTATGCGCTCGGTGGTGGTTGTGAATTGGCAATGATGTGCGACATCATC
ATCGCCAGTGAAAACGCGCAGTTCGGACAACCGGAAATCAATCTGGGCATCATTCCEGGT
GCTGGTGGCACCCAACGGCTGACCGCGCCCTTGCCCGTATCGCGCAATGGAATTGATC
CTGACCGGCGCGACCATCAGTGCTCAGGAAGCTCTCGCCACGGCCTGGTGTGCCGGGTC
TGCCCGCCTGAAAGCCTGCTCGATGAAGCCCGTGGGATCGCGCAAACCATTGCCACCAAA
TCACCACTGGCTGTACAGTTGGCGAAAGAGGCAGTCCGTATGGCCGCGGAAACCACTGTG
CGCGAGGGGTTGGCTATCGAGCTGCGTAACTTCTATCTGCTGTTTGCCAGTGCTGACCAA
AAAGAGGGGATGCAGGCATTTATCGAGAAACGCGCTCCCAACTTCAGTGGTCGTTGA
(SEQ ID NO:40)

Figure 30

MSEESLVLSTIEGPIAILTLNRPQALNALS PALIDDLIRHLEACDADD TIRVIIITGAGR
AFAAGADIKAMANATPIDMLTSGMIARWARIAAVRKPVIAAVNGYALGGGCELAMMCDII
IASENAQFGQPEINLGIIPGAGGTQRLTRALGPYRAMELILTGATISAEALAHGLVCRV
CPPESSLDEARRIAQTIATKSPLAVQLAKEAVRMAAETTVREGLAIELRNFYLLFASADQ
KEGMQAFIEKRAPNFSGR (SEQ ID NO:41)

Figure 31

GGCGTAATCCGACCGGCAGGTTAGGGTCTTCTACTGGGGTCAAGGCGCGTCTCCTTTTGG
TGGCGCGAGCAACCCGGCTTTTCCTGGCTTCAATGTACCATAGAGCGGTACTTCGTGCA
ACGGGCGTGGTACAATCGAGAGCAACCTTTCGCAAAAGCTATCCAATCCTGCACACGTGC
ATCTGTTACAGGGTATTATTGTTCGGCAAACGACAGTCCTGTCTGTTTATGTACAAGGAGAT
CAACGTATGAGTGAAGAGTCTCTGGTTCTCAGCACAAATTGAAGGCCCCATGGCCATCCTC
ACCCTCAATCGCCCCCAGGCCCTCAATGCGCTCAGTCCGGCCTTGATTGATGACCTCATT
CGCCATTTAGAAGCCTGCGATGCCGATGACACAATCGGCGTGATCATTATCACCGGCGCC
GGACGGGCATTTGCTGCCGGCGCTGATATCAAAGCGATGGCCAATGCCACGCCTATTGAT
ATGCTCACCAGTGGCATGATTGCGCGCTGGGCACGCATCGCCGCGGTGCGCAAACGGGTG
ATTGCTGCCGTGAATGGGTATGCGCTCGGTGGTGGTTGTGAATTGGCAATGATGTGCGAC
ATCATCATCGCCAGTGAAAACGCGCAGTTCGGACAACCGGAAATCAATCTGGGCATCATT
CCCGGTGCTGGTGGCACCCAACGGCTGACCCGCGCCCTTGGCCCGTATCGCGCAATGGAA
TTGATCCTGACCGGCGCGACCATCAGTGCTCAGGAAGCTCTCGCCACGGCCTGGTGTGC
CGGTCTGCCCGCCTGAAAGCCTGCTCGATGAAGCCCGTGGATCGCGCAAACCATTGCC
ACCAAATCACCAGTGGCTGTACAGTTGGCGAAAGAGGCAGTCCGTATGGCCGCCGAAACC
ACTGTGCGCGAGGGGTGGCTATCGAGCTGCGTAACTTCTATCTGCTGTTTGCCAGTGCT
GACCAAAAAGAGGGGATGCAGGCATTTATCGAGAAACGCGCTCCCAACTTCAGTGGTGGT
TGATCAGCGCGAGAACATGGCAGCAGGGGCAATACCTGCACGTACTGCCTCCTGCCGCA
TACTACCAGATGATCGAGCAGTAAAGGGTAAATACTCTATCAATCTGGCCAGATAAGCGG
TTGGGTAAACAACGCAATGCTCCAAAGGAGACGATCATGGACATACAGAGCGATTGCGAT
CTCTCGAACGCGAAAATGCT (SEQ ID NO:42)

Figure 32

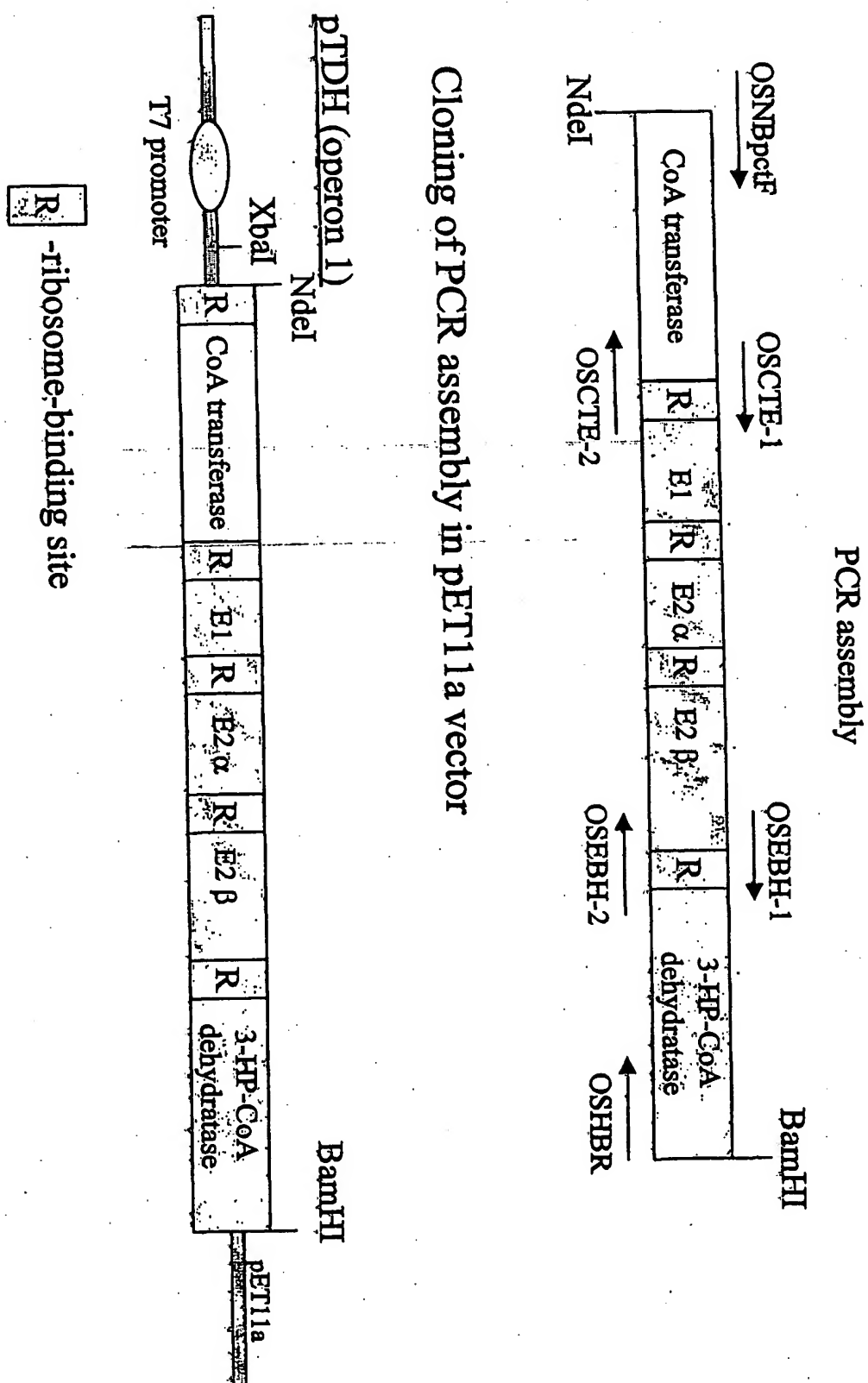
SEQ ID NO:40	1	-----atgagtga-----agagt-----
SEQ ID NO:43	1	-----atgacgta-----cgaaa-----
SEQ ID NO:44	1	atggccgccctgcgtgt-----cctgctgtcctgcgccgcggcc
SEQ ID NO:45	1	atggcgccctgcgtgctctgctgccagagc-----
SEQ ID NO:40	14	-----ct-----ctg-----gttctc-agcacaattgaa
SEQ ID NO:43	14	-----cc-----atc-----ctggctcgagcgc---gat
SEQ ID NO:44	41	cgctgaggccc-----ccg-----gttcgc-tgtccgcctgg
SEQ ID NO:45	33	-----ctgcaactcgctgttgtccccagttcgc-tgccagaattc
SEQ ID NO:40	37	ggccccatcgcc-----atcctcacc-----
SEQ ID NO:43	34	cagcgagttggc-----attatcacg-----
SEQ ID NO:44	73	cgtcccttcgctcgggtgctaactttgagtacatcatcgcaaaaaag
SEQ ID NO:45	73	cgcgcttcgctcgggtgctaactttcagtacatcatcacg-----
SEQ ID NO:40	58	-----c-----
SEQ ID NO:43	55	-----c-----
SEQ ID NO:44	123	agggagaataaacaccgtggggttgatccaac-----
SEQ ID NO:45	115	-----gaaaagaaggaaagaata
SEQ ID NO:40	59	-----tcaatcgccccaggccctcaatgcgctc
SEQ ID NO:43	56	-----tgaaccgtcccaggcactgaacgcgctc
SEQ ID NO:44	155	-----tgaaccgccccaggccctcaatgcactt
SEQ ID NO:45	134	gcagcgtggggctgatccagttgaaccgtcccaggcactcaatgcactt
SEQ ID NO:40	88	agtccggccttgattgatgacctattc--gccatttagaagcctgcgat
SEQ ID NO:43	85	a--acagccagg--tgatgaacgaggtc--acca--gcgctgcaaccgaa
SEQ ID NO:44	184	tgcatggccttgattgacgagctcaaccaggccctgaaga--tcttcgag
SEQ ID NO:45	184	tgcaatggactgattgaggagctcaacc--aagcactggagacctttgag
SEQ ID NO:40	136	---gccgatgacaca---atccgcgtgatcattatcacccggcgccggagc
SEQ ID NO:43	127	ctggacgatgacccggacatttggggcgatcatcatcacccggttcggccaa
SEQ ID NO:44	232	---gaggacccggcc---gttggggcattgtcctcacccggcggggataa
SEQ ID NO:45	232	---gaagatcccgt---gtgggcgccattgtgctcactggtggggagaa
SEQ ID NO:40	180	ggcatttgcgtccggcgctgatatcaaagcgatggccaa-----tgcc
SEQ ID NO:43	177	agcgtttgcgcgggagccgacatcaaagaaatggccga-----cctg
SEQ ID NO:44	276	ggcctttgcagctggagctgatatcaaggaaatgcagaacctgagttcc
SEQ ID NO:45	276	ggcctttgcagccggagctgacatcaaggaaatgcagaa-----ccgg
SEQ ID NO:40	223	acgcctattgatatgctcaccagtgccatgattgcgcgc---tgggcacg
SEQ ID NO:43	220	acgttcgcccagcgcgttcacccgacgttcttcgccacc---tggggcaa
SEQ ID NO:44	326	aggactgtt-----actccagcaagttcttgaagcac---tggggcca
SEQ ID NO:45	319	acatttcagga-ctgttactca--ggcaagttcctgagccactgggacca
SEQ ID NO:40	270	catcgccgcggtgcgcgcaaacccggtgattgctgcccgtgaatgggtatgcgc
SEQ ID NO:43	267	gctggccgcggtgcgcaccccgacgatcgccgcggtggcgggatacgcg
SEQ ID NO:44	366	cctcaccaggtcaagaagccagtcacgtgctgtcaatggctatccgt
SEQ ID NO:45	366	tatcaccgggatcaagaaaccggtcatcgccggtgtcaatggctatgctc
SEQ ID NO:40	320	tcggtggtggttgtgaattggcaatgatgtgcgacatcatcatcgccagt
SEQ ID NO:43	317	tcggcggtggtgcgagctggcgatgatgtgcgacgtgctgatcgccgcc
SEQ ID NO:44	416	ttggcggggctgtgagcttgccatgatgtgtgatcatcatcgccgt
SEQ ID NO:45	416	ttggtggggctgtgaacttgccatgatgtgcgatatcatcatgctggt

SEQ ID NO:40	370	gaaaacgcgcagttcgggacaaccggaaatcaatctggggcatcattcccg
SEQ ID NO:43	367	gacaccgcgaagttcgggacagcccagagataaagctgggctgctgccagg
SEQ ID NO:44	466	gagaaggcccagtttgcacagccggagatcttaataggaacctcccagg
SEQ ID NO:45	466	gagaaagcccagtttggacagccagaaatcctcctggggaccatcccagg
SEQ ID NO:40	420	tgctggtggcacccaacggctgacccgcgccttggcccgatcgcgcaa
SEQ ID NO:43	417	catgggcggtctccagcggctgacccgggctatcggcaaggctaaggcga
SEQ ID NO:44	516	tgcaggcggcacccagagactcaccctgctgttgggaagtgcgtggagc
SEQ ID NO:45	516	tgcagggggcactcagagactcacccgagcagtcggcaaatcactagcaa
SEQ ID NO:40	470	tggaattgatcctgaccggcgcgaccatcagtgcaggaagctctcgcc
SEQ ID NO:43	467	tggacctcatcctgaccggcgcaecatggacgcgcggcaggc-cgagcg
SEQ ID NO:44	566	tggagatggtcctcaccggtgacgcgatctcagcccaggacgc-caagca
SEQ ID NO:45	566	tggagatggtcctcactggtgaccgaatttcagcacaggatgc-caagca
SEQ ID NO:40	520	ca-c-ggcctggtgtgcccgggtctgccgcctgaaagcctgctcgatgaa
SEQ ID NO:43	516	cagc-ggtctggtttcacgggtggtgcccggcgcgacttgcagaccgaa
SEQ ID NO:44	615	ag-caggtcttgcagcaagatttgcctgttgagacactgggtggaagaa
SEQ ID NO:45	615	ag-caggtcttgaagcaagattttcccgttgaacactgggtggaagag
SEQ ID NO:40	568	gcccgctcggtatcgcgcaaacattgccaccaaaccactgggtgtata
SEQ ID NO:43	565	gccagggccactgccacgacatttcgcagatgtcgccctcgggcgcccg
SEQ ID NO:44	664	gccatccagtgtgcagaaaaaattgccagcaattctaaaattgtagtagc
SEQ ID NO:45	664	gccatccaatgtgcagaaaagatcgccaacaattccaagatcatagtagc
SEQ ID NO:40	618	gttggcgaaagaggcagtcctgatggcgccgaaaccactgtgcgcgagg
SEQ ID NO:43	615	gatggccaaggaggccgtcaaccgggctttcgaaatccagtttgtccgagg
SEQ ID NO:44	714	gatggccaagaatcagtgaatgcagcttttgaaatgacattaacagaag
SEQ ID NO:45	714	catggcgaaagaatctgtgaatgcagcctttgaaatgacgttaacagaag
SEQ ID NO:40	668	ggttggctatcgagctgcgttaacttctatctgctgtttgccagtgcgtgac
SEQ ID NO:43	665	ggctgctctacgaacgcgggcttttccattcggctttcgcgaccgaagac
SEQ ID NO:44	764	gaagtaagttggagaagaaactctttattcaacctttgccactgatgac
SEQ ID NO:45	764	gaaataagctggagaagaagctcttctattccacctttgccactgatgac
SEQ ID NO:40	718	caaaaagaggggatgcaggcatttatcgagaaacgcgctcccaacttcag
SEQ ID NO:43	715	caatccgaagggtatggcagcgttcacgcagaaacgcgctcccagttcac
SEQ ID NO:44	814	cggaaagaagggatgaccgcgtttgtggaaaagagaaaggccaacttcaa
SEQ ID NO:45	814	cggagagaagggatgtctgcctttgtggagaaaaggaaggccaacttcaa
SEQ ID NO:40	768	tggtcgttga
SEQ ID NO:43	765	ccaccgatga
SEQ ID NO:44	864	agaccagtga
SEQ ID NO:45	864	agaccactga

Figure 33

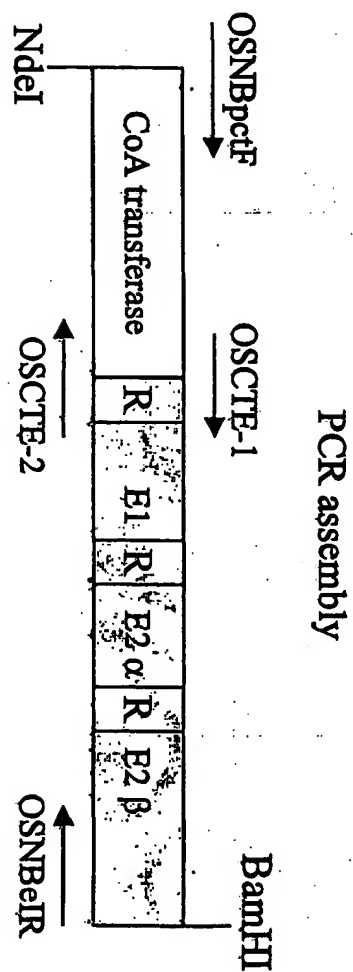
SEQ ID NO:41	1 -mseeslv-----lstiegp-----
SEQ ID NO:46	1 -mtyetil-----ver-dqr-----
SEQ ID NO:47	1 -maalrvl-----lscargplrppvrpcpawrpfasganfeyiiaekrg
SEQ ID NO:48	1 maalrallpracnsllspvrcepfrrfasganfqyiitekkgknss----
SEQ ID NO:41	15 ----iailtlnrpqalnalspaliddlirhleacdaddtirvliitgagr
SEQ ID NO:46	14 ----vgiitlnrpqalnalsqvmnevtsaateldddpdigaiiitgsak
SEQ ID NO:47	43 knntvgliqlnrpkalnalcldglidelnqalkifeedpavgaivltggdk
SEQ ID NO:48	47 ----vgliqlnrpkalnalcnglieelnqaletfeedpavgaivltggek
SEQ ID NO:41	61 afaagadikamanatpidmltsgmiarwariaavrkpviaavngyalggg
SEQ ID NO:46	60 afaagadikemadltfadaftadffatwgklaavrtptiaavagyalggg
SEQ ID NO:47	93 afaagadikemqnlstfqcyskflkhwdhltqvkkpviaavngyafggg
SEQ ID NO:48	93 afaagadikemqnrftqdcysgkflshwdhitrikkpviaavngyalggg
SEQ ID NO:41	111 celammcdiiiasenaqfgqpeinlgiipgaggtqrltralgpypyrameli
SEQ ID NO:46	110 celammcdvliiaadtakfgqpeiklgvlpmggsqrltraigkakamdli
SEQ ID NO:47	143 celammcdiiyagekaqfaqpeiligtipgaggtqrltravgkslamemv
SEQ ID NO:48	143 celammcdiiyagekaqfgqpeiligtipgaggtqrltravgkslamemv
SEQ ID NO:41	161 ltgatisaqealahglvcrvcppeslldearriaqtiatksplavqlake
SEQ ID NO:46	160 ltgrtmdaaeaersglvsrvvpaddlltearatattisqmsasaarmake
SEQ ID NO:47	193 ltgdrisadqakqaglvskicpvetlveeaiqcaekiasnskiivvmake
SEQ ID NO:48	193 ltgdrisadqakqaglvskifpvetlveeaiqcaekiannskiivvmake
SEQ ID NO:41	211 avrmaaettvreglaieiInfyllfasadqkegmqafiekrapnfsgr
SEQ ID NO:46	210 avnrafesslsegliyerlrfhsafatedqsegmaafiekrapqfthr
SEQ ID NO:47	243 svnaafemtltegskleklkfystfatddrkegmtafvekrkanfkdk
SEQ ID NO:48	243 svnaafemtltegnkleklkfystfatddrregmsafvekrkanfkdk

Figure 34



Cloning of PCR assembly in pET11a vector

Figure 35A



Cloning of PCR assembly in pET11a vector

pTD

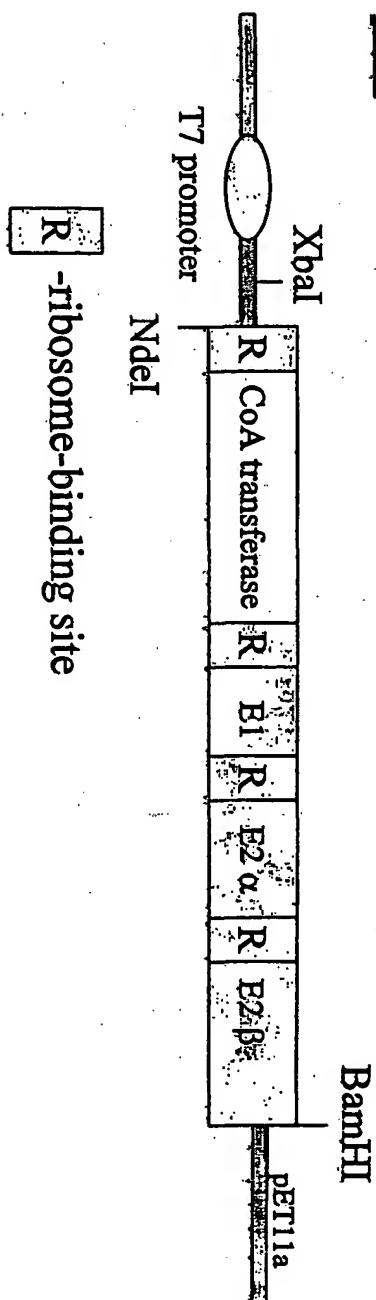


Figure 35B

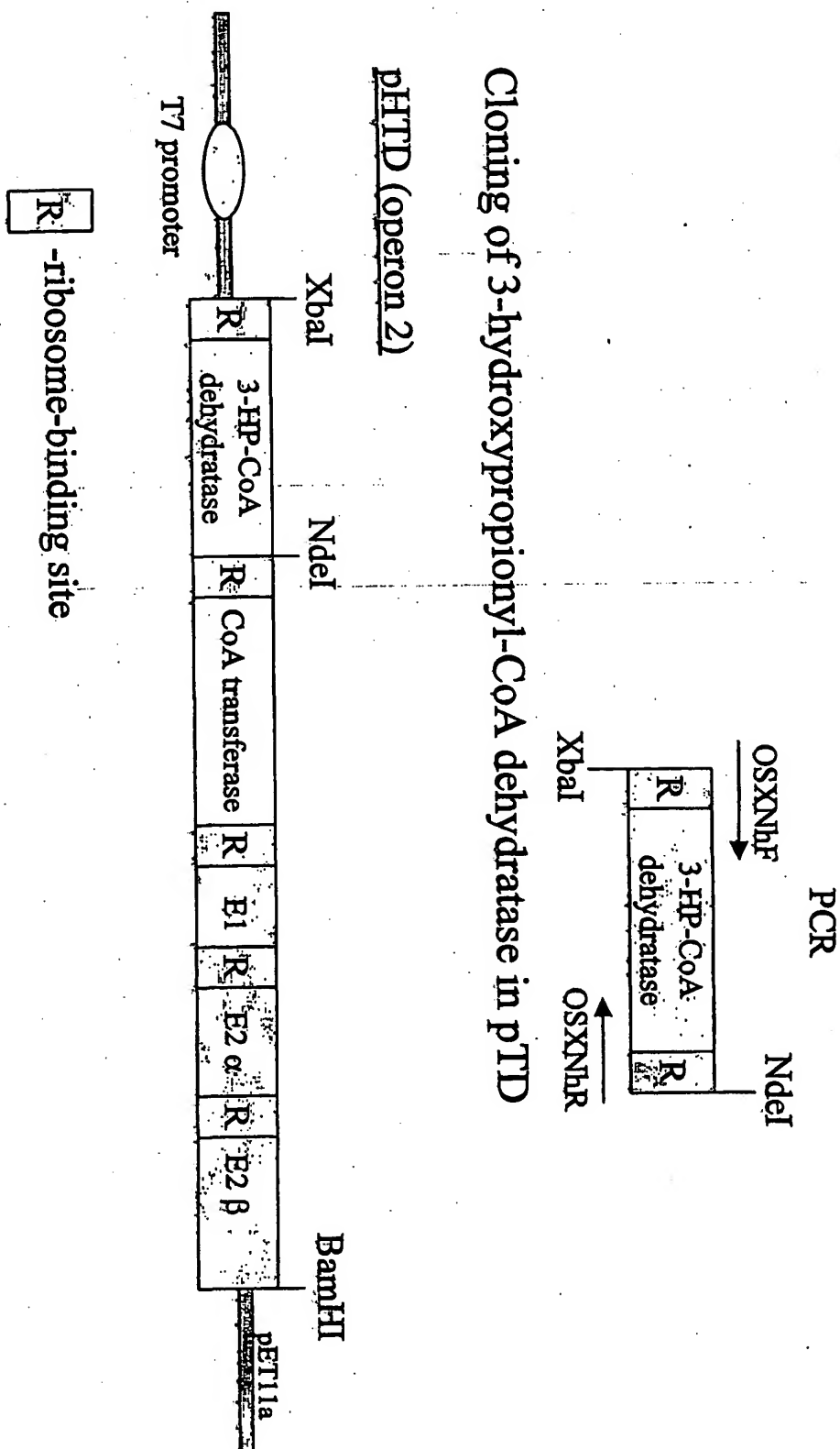


Figure 36A

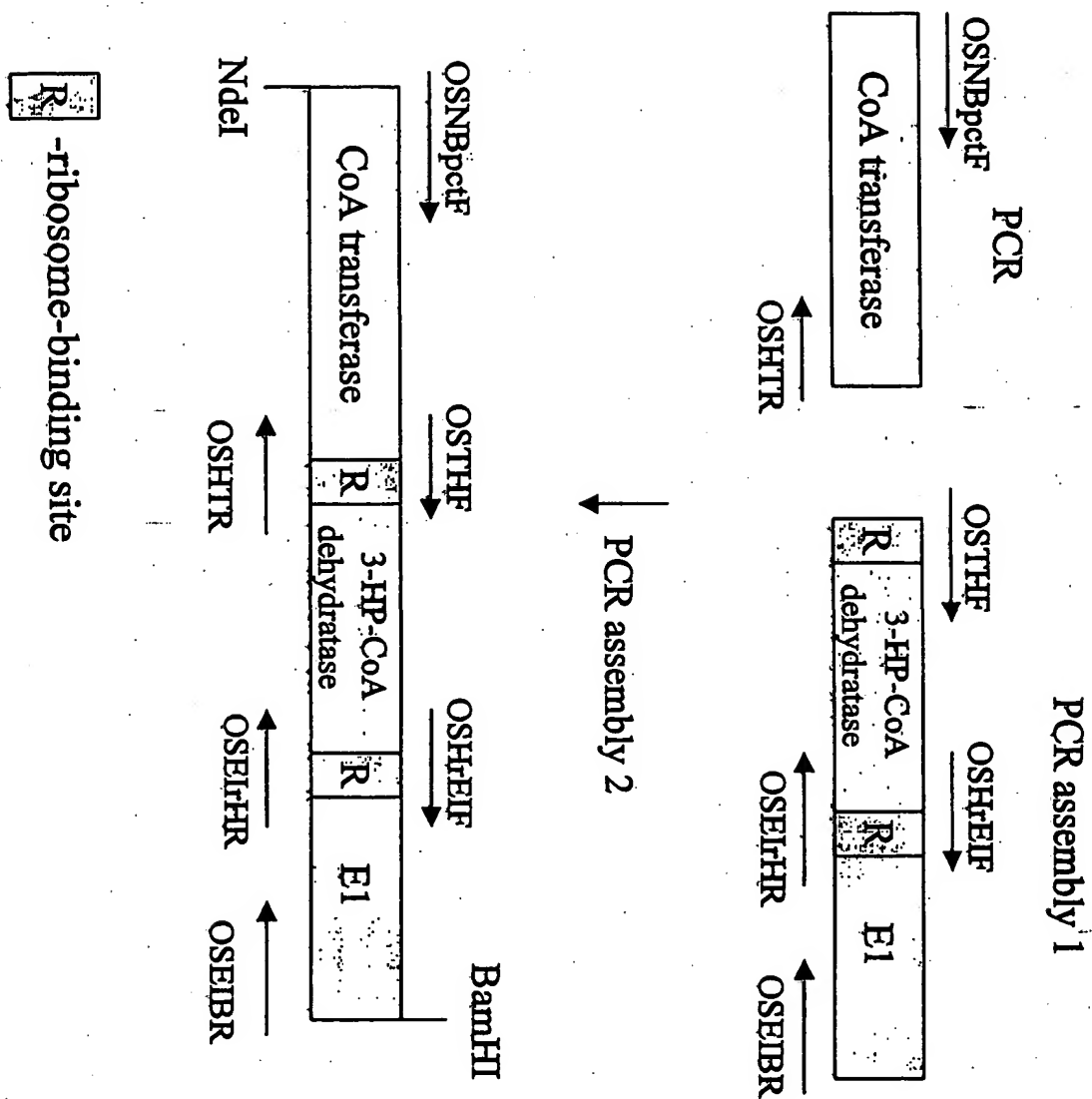


Figure 36B

Cloning of PCR assembly 2 in pET11a vector

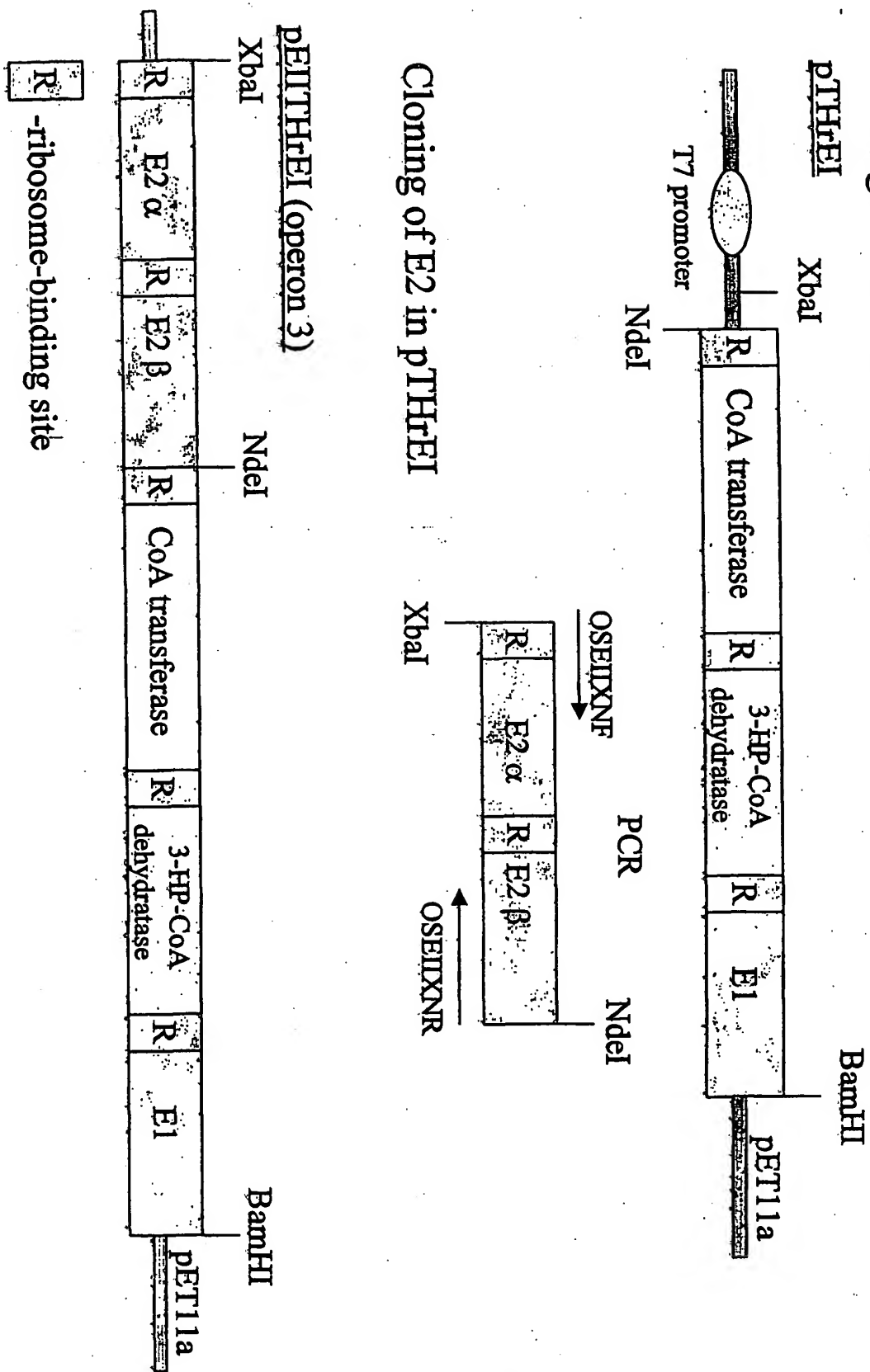


Figure 37A

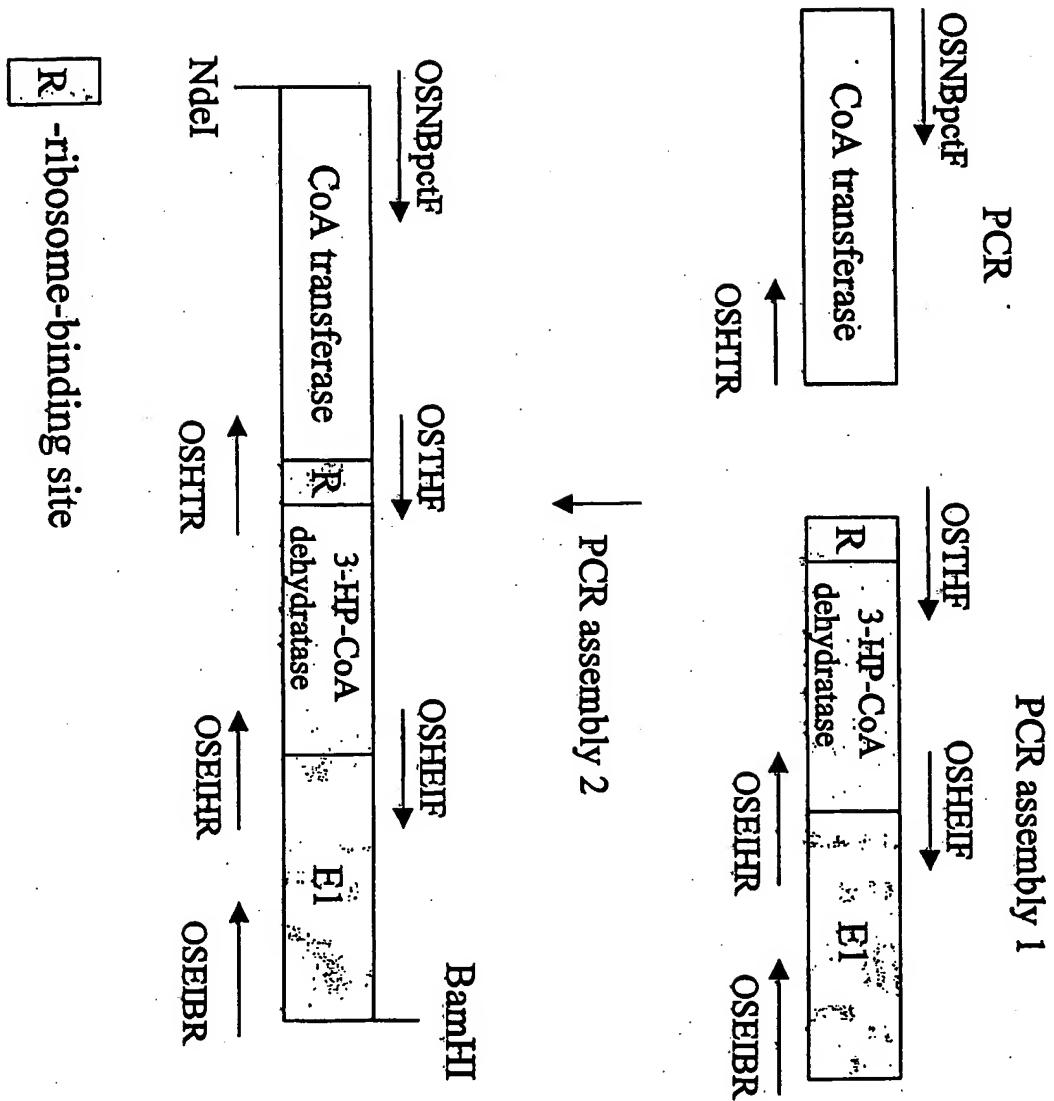


Figure 37B

Cloning of PCR assembly 2 in pET11a vector

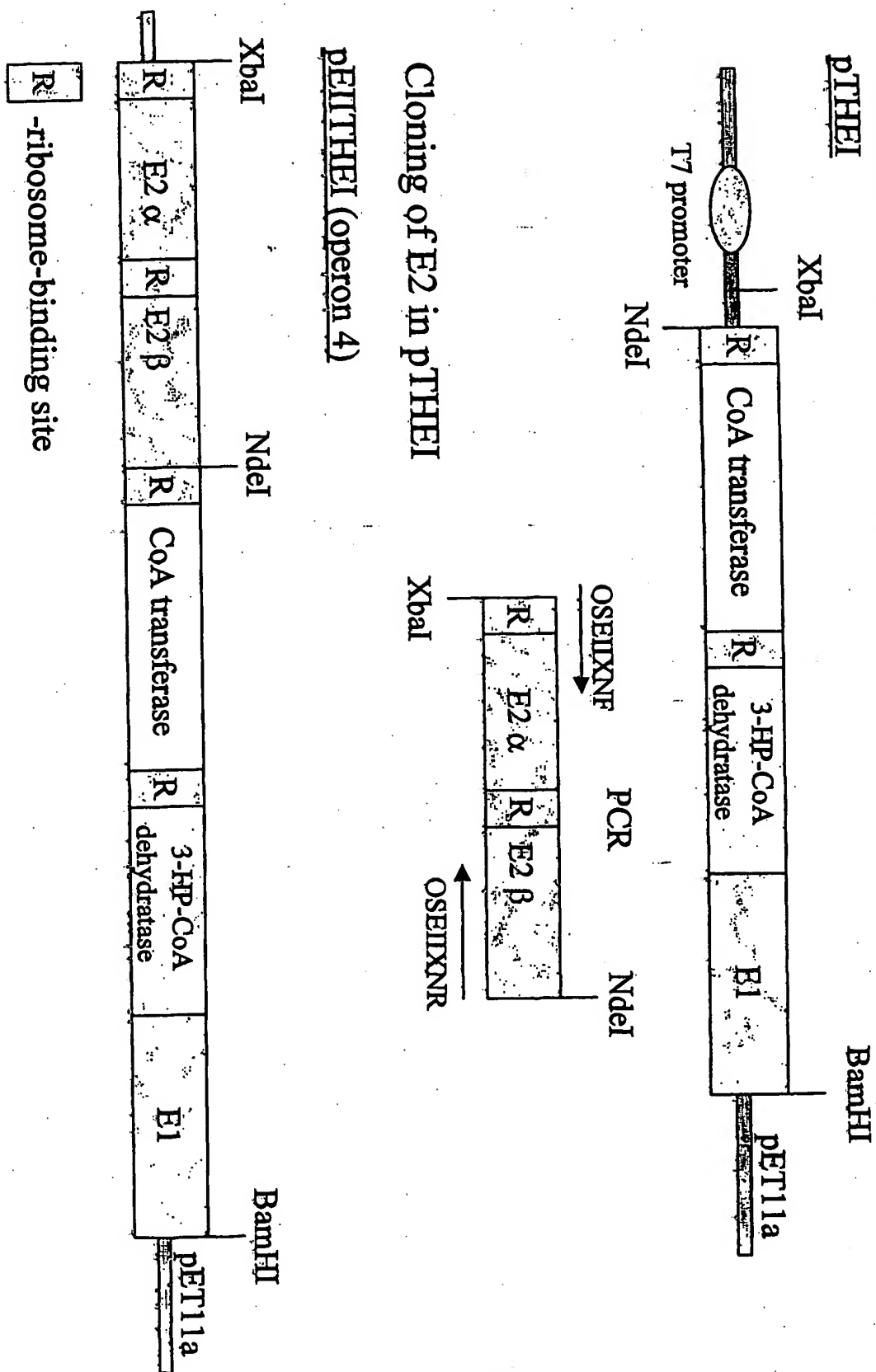


Figure 38A

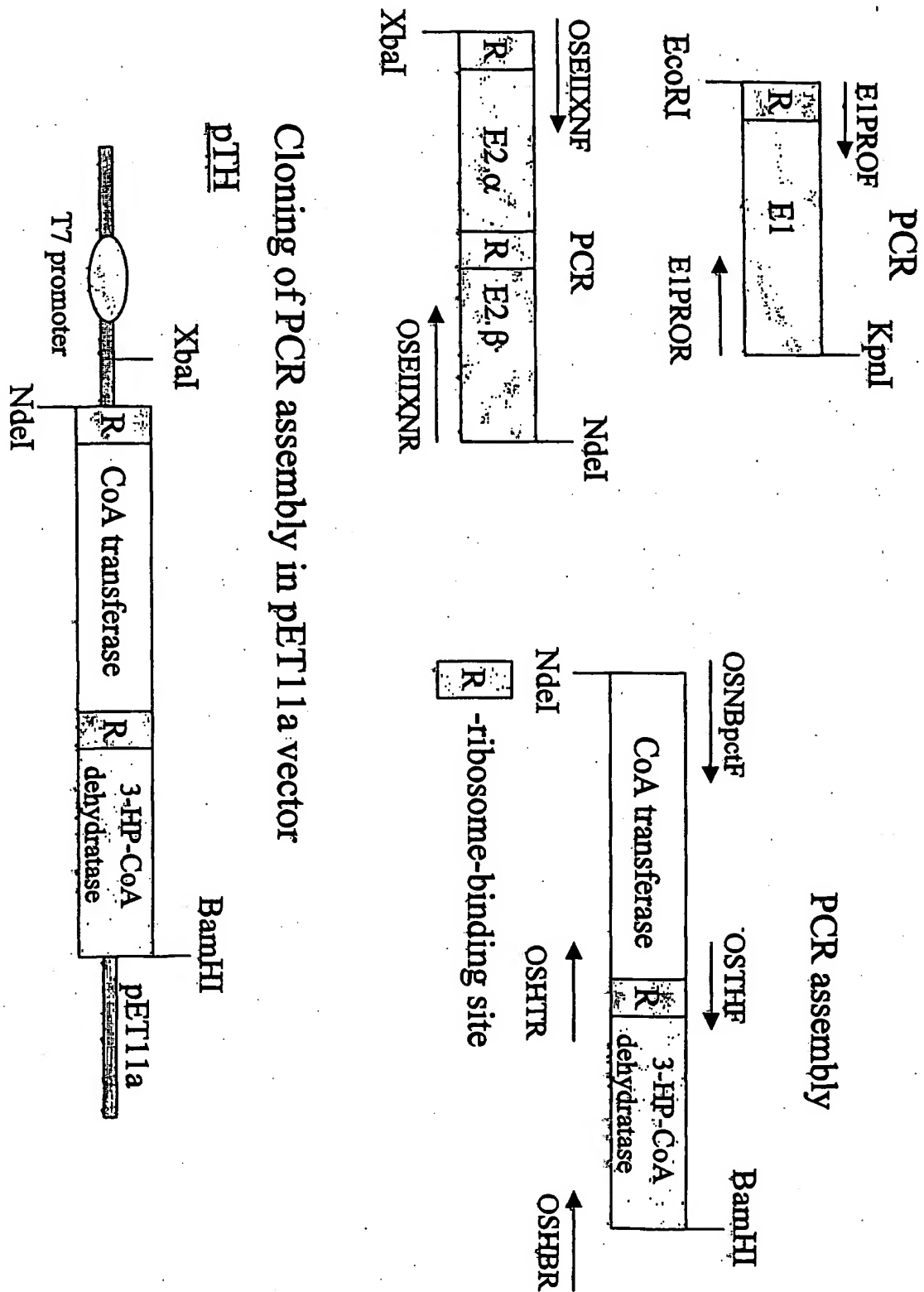
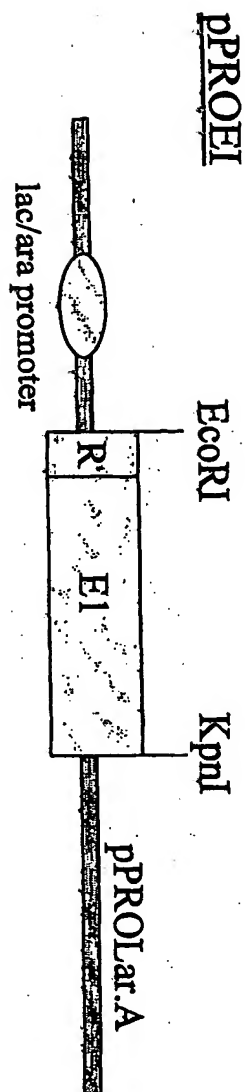


Figure 38B

Cloning of E1 gene separately in pPROLar.A vector



Cloning of E2 in pTH

pTH (operon 5)

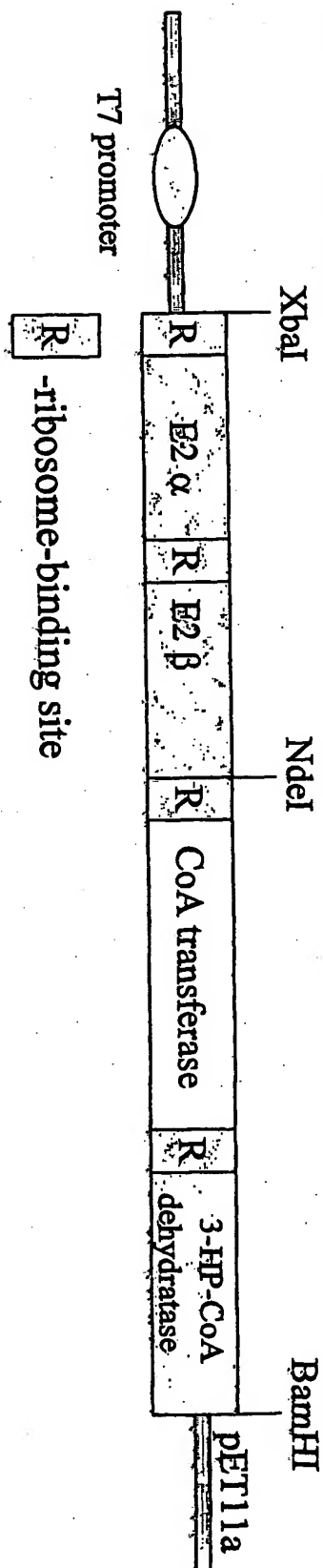


Figure 39

ATGATCGACACTGCGCCCCCTTGCCCCACCAAGGGCGCGCGCTCTAATCCGATTTCGGGAT
CGAGTTGATTGGGAAGCTCAGCGCGCTGCTGCGCTGGCAGATCCCGGTGCCTTTCATGGC
GCGATTGCCCCGACAGTTATCCACTGGTACGACCCACAACACCATTGCTGGATTTCGCTTC
AACGAGTCTAGTCAGCGTTGGGAAGGGCTGGATGCCGCTACCGGTGCCCTGTAAACGGTA
GACTATCCCCCGGATTATCAGCCCTGGCAACAGGCGTTTGTATGATAGTGAAGCGCCGTTT
TACCGCTGGTTTAGTGGTGGGTTGACAAATGCCTGCTTTAATGAAGTAGACCGGCATGTC
ATGATGGGCTATGGCGACGAGGTGGCCTACTACTTTGAAGGTGACCGCTGGGATAACTCG
CTCAACAATGGTCGTGGTGGTCCGTTGTCCAGGAGACAATCACGCGGCGGGCGCCTGTTG
GTGGAGGTGGTGAAGGCTGCGCAGGTGTTGCGTGATCTGGGCTGAAGAAGGGTGATCGG
ATTGCTCTGAATATGCCGAATATTATGCCGAGATTTATTATACGGAAGCGGCAAAACGA
CTGGGTATTCTGTACACGCCGGTCTTCGGTGGCTTCTCGGACAAGACTCTTTCGACCGT
ATTCACAATGCCGGTGCACGAGTGGTGATTACCTCTGATGGTGCGTACCGCAACGCGCAG
GTGGTGCCCTACAAAGAAGCGTATACCGATCAGGGCGCTCGATAAGTATATTCCGGTTGAG
ACGGCGCAGGCGATTGTTGCGCAGACCTGGCCACCTTGCCCCGACTGAGTCGCAGCGC
CAGACGATCATCACCGAAGTGGAGGCCGCACTGGCCGGTGAGATTACGGTTGAGCGCTCG
GACGTGATGCGTGGGGTTGGTCTGCCCTCGCAAAGCTCCGCGATCTTGATGCAAGCGTG
CAGGCAAAGGTGCGTACAGTACTGGCGCAGGCGCTGGTTCGAGTCGCCGCCCGGGTTGAA
GCTGTGGTGGTTGTGCGTCATACCGTTCAGGAGATTTTGTGGAACGAGGGGCGAGATCGC
TGGAGTCACGACTTGCTGGATGCTGCGCTGGCGAAGATTCTGGCCAATGCGCGTGCTGCC
GGCTTTGATGTGCACAGTGAGAATGATCTGCTCAATCTCCCCGATGACCAGCTTATCCGT
GCGCTCTACGCCAGTATTCCCTGTGAACCGGTTGATGCTGAATATCCGATGTTTATCATT
TACACATCGGGTAGCACCGGTAAGCCCCAAGGGTGTGATCCACGTTACCGCGGTTATGTC
GCCGGTGTGGTGCACACCTTGCGGGTCAGTTTTGACGCCGAGCCGGGTGATACGATATAT
GTGATCGCCGATCCGGGCTGGATCACCGGTTCAGAGCTATATGCTCACAGCCACAATGGCC
GGTCGGCTGACCGGGGTGATTGCCGAGGGATCACCGCTCTTCCCCCTCAGCCGGGGGTTAT
GCCAGCATCATCGAGCGCTATGGGGTGCAGATCTTTAAGGCGGGTGTGACCTTCCTCAAG
ACAGTGATGTCCAATCCGCAGAATGTTGAAGATGTGCGACTCTATGATATGCACTCGCTG
CGGGTTGCAACCTTCTGCGCCGAGCCGGTCAGTCCGGCGGTGCAGCAGTTTGGTATGCAG
ATCATGACCCCGCAGTATATCAATTCGTAAGTGGGCGACCGAGCACGGTGGAAATGTCTGG
ACGCATTTCTACGGTAATCAGGACTTCCCGCTTCGTCCCGATGCCCATACCTATCCCTTG
CCCTGGGTGATGGGTGATGTCTGGGTGGCCGAACTGATGAGAGCGGGACGACGCGCTAT
CGGGTCGCTGATTTTCGATGAGAAGGGCGAGATTGTGATTACCGCCCCGTATCCCTACCTG
ACCCGCACACTCTGGGGTGTGTCGCCGGTTTCGAGGCGTACCTGCGCGGTGAGATTCCG
CTGCGGGCCTGGAAGGGTGTGTCGAGCGTTTCGTCAAGACCTACTGGCGACGTGGGCCA
AACGGTGAATGGGGCTATATCCAGGGTGATTTTGCCATCAAGTACCCCGATGGTAGCTTC
ACGCTCCACGGACGCCCTGACGATGTGATCAATGTGTGCGGGCCACCGTATGGGCACCGAG
GAGATTGAGGGTGCCATTTTGCCTGACCGCCAGATCACGCCCGACTCGCCCGTCCGTAAT
TGTATTGTGGTCCGTGCGCCGACCGTGAGAAGGGTCTGACCCCGGTTGCCCTTCATTCAA
CCTGCGCCTGGCCGTATCTGACCGGCGCCGACCGGCGCGCTCTCGATGAGCTGGTGGCT
ACCGAGAAGGGGGCGGTGAGTGTCCAGAGGATTACATCGAGGTGAGTGCCTTTCCCGAA
ACCCGCAGCGGGAAGTATATGCGGCGCTTTTTCGCAATATGATGCTCGATGAACCACTG
GGTGATACGACGAGTTCGCAATCCTGAAGTGCTCGAAGAGATTGCAGCCAAGATCGCT
GAGTGGAAACGCCGTGAGCGTATGGCCGAAGAGCAGCAGATCATCGAAGCTATCGCTAC
TTCCGGATCGAGTATCACCCACCAACGGCCAGTGGCGGTAAACTCGCGGTAGTGACGGTG
ACAAATCCGCGGGTGAACGCACTGAATGAGCGTGCGCTCGATGAGTTGAACACAATTGTT
GACCACCTGGCCCGTCTGAGGATGTTGCCGCAATTGTCTTACCGGACAGGGCGCCAGG
AGTTTTGTGCGCGGCGCTGATATTGCCAGTTGCTCGAAGAGATTATACGGTTGAAGAG
GCAATGGCCCTGCCGAATAACGCCCATCTTGCTTTCGCAAGATTGAGCGTATGAATAAG

CCGTGTATCGCGGGGATCAACGGTGTGGCGCTGGGTGGTGGTCTGGAATTCGCCATGGCC
TGCCATTACCGGGTTGCCGATGTCTATGCCGAATTCGGTCAGCCAGAGATTAATCTGCGC
TTGCTACCTGGTTATGGTGGCACGCGCTTGCCGCGCCTGTTGTACAAGCGCAACAAC
GGCACCGGTCTGCTCCGAGCGCTGGAGATGATTCTGGGTGGGCGTAGCGTACCGGCTGAT
GAGGCGCTGAAGCTGGGTCTGATCGATGCCATTGCTACCGGCGATCAGGACTCACTGTCTG
CTGGCATGCGCGTTAGCCCGTGCCGCAATCGGCGCCGATGGTCAGTTGATCGAGTCGGCT
GCGGTGACCCAGGCTTTCCGCCATCGCCACGAGCAGCTTGACGAGTGGCGCAAACCAGAC
CCGCGCTTTGCCGATGACGAAC TGCGCTCGATTATCGCCCATCCACGTATCGAGCGGATT
ATCCGGCAGGCCCATACCGTTGGGCGCGATGCGGCAGTGCATCGGGCACTGGATGCAATC
CGCTATGGCATTATCCACGGCTTCGAGGCGGGTCTGGAGCACGAGGCGAAGCTCTTTGCC
GAGGCAGTGGTTGACCCGAACGGTGGCAAGCGTGGTATTCGCGAGTTCTTCGACCGCCAG
AGTGCGCCGTTGCCAACCCGCGACCATTGATTACACCTGAACAGGAGCAACTCTTGCGC
GATCAGAAAGAACTGTTGCCGGTTGGTTACCCCTTCTTCCCGGTGTTGACCGGATTCCG
AAGTGGCAGTACGCGCAGGCGGTTATTCTGTGATCCGGACACCGGTGCGGCGGCTCACGGC
GATCCCATCGTGGCTGAAAAGCAGATTATTGTGCCGGTGGAAACGCCCCCGGCCAATCAG
GCGCTGATCTATGTTCTGGCCTCGGAGGTGAACTTCAACGATATCTGGGCGATTACCGGT
ATTCCGGTGTACGGTTTGATGAGCACGACCGCGACTGGCAGGTTACCGGTTACAGGTGGC
ATCGGCCTGATCGTTGCGCTGGGTGAAGAGGCGCGACGCGAAGGCCGGCTGAAGGTGGGT
GATCTGGTGGCGATCTACTCGGGCAGTCGGATCTGCTCTCACCGCTGATGGGCCTTGAT
CCGATGGCCGCGGATTTCGTATCCAGGGGAACGACACGCCAGATGGATCGCATCAGCAA
TTTATGCTGGCCAGGCCCGCAGTGTCTGCCCATCCCAACCGATATGTCTATCGAGGCA
GCCGGCAGCTACATCCTCAATCTCGGTACGATCTATCGCGCCCTCTTACGACGTTGCAA
ATCAAGGCCGGACGCACCATCTTTATCGAGGGTGGCGGACCGGTACCGGTCTGGACGCA
GCGCGCTCGGCGGCCCGGAATGGTCTGCGCGTAATTGGAATGGTCAGTTCTGTCGTACGT
GCGTCTACGCTGCTGGCTGCGGGTGCCACGCTGCGATTAAACGGTAAAGACCCGGAGGTT
GCCGATTGTTTACGCGCGTGCCCGAAGATCCATCAGCCTGGGCGAGCTGGGCGATTATGTG
GTCTCGCACGCGGGCGAGACGGCCTTCCGCGCAGTTTCCAGCTTCTCGGCGAGCCACGC
GATGGTCACATTCCGACGCTCACATTCTACGGTGCCACCAGTGGCTACCACTTCACCTTC
CTGGGTAAAGCCAGGGTCAGCTTCGCCGACCGAGATGCTGCGGCGGGCCAATCTCCGCGCC
GGTGAGGCGGTGTTGATCTACTACGGGTTGGGAGCGATGACCTGGTAGATAACCGGCGGT
CTGGAGGCTATCGAGGCGGCGCGCAAATGGGAGCGCGGATCGTCGTGTTACCGTCAGC
GATGCGCAACGCGAGTTTGTCTCTCGTTGGGCTTCGGGGCTGCCCTACGTGGTGTCTGTC
AGCCTGGCGGAAC TCAAACGGCGCTTCGGCGATGAGTTTGAGTGGCCGCGCACGATGCCG
CCGTTGCCGAACGCGCCGCGCAGGACCGCAGGGTCTGAAAGAGGCTGTCCGCCGCTTCAAC
GATCTGGTCTTCAAGCCGCTAGGAAGCGCGGTGGTGTCTTCTTGCGGAGTGCCGACAAT
CCGCGTGGCTACCCCGATCTGATCATCGAGCGGGCTGCCACGATGCACTGGCGGTGAGC
GCGATGCTGATCAAGCCCTTACCGGACGGATTGTCTACTTCGAGGACATTGGTGGGCGG
CGTTACTCCTTCTTCGCACCGCAAATCTGGGTGCGCCAGCGCCGATCTACATGCCGACG
GCACAGATCTTTGGTACGCACCTCTCAAATGCGTATGAAATTCTGCGTCTGAATGATGAG
ATCAGCGCCGGTCTGCTGACGATTACCGAGCGGCGAGTGGTGGCGTGGGATGAACTAACC
GAAGCACATCAGGCGATGTGGGAAAATCGCCACACGGCGGCCACTTATGTGGTGAATCAT
GCCTTACCACGTCTCGGCCTAAAGAACAGGGACGAGCTGTACGAGGCGTGGACGGCCGGC
GAGCGGTAG (SEQ ID NO:129)

Figure 40

SEQ ID NO:39	1	-----midtaplappprsrnpirdrvdwe
SEQ ID NO:130	1	mglpeervrsgsgsrqgeeagaggrarswsp--ppevrsahvpqlqryr
SEQ ID NO:131	1	-----mslelkekeselpfdeqiind
		PL PP RS P
SEQ ID NO:39	26	aqraaaladpgafhgaiartvihwydpqhhcwifnessqrweglidaatg
SEQ ID NO:130	49	elhrrsveeprefwgdiake-fywktpcpgpflryn-----
SEQ ID NO:131	22	kwr-----kytpidayfkfhrqtvenlesf--wesv-----
		R P F G I A T I W Y P H R NES WE
SEQ ID NO:39	76	apvtvdypadyqpwwqafddseap-fyrwfsaggltfnacfnvdrhvm-mg
SEQ ID NO:130	84	-----fdvtkgkifiewmkgattnicynvldrnvhckk
SEQ ID NO:131	52	-akelew---fkpwdkvldasnpp-fykwfvggrrlnlsylavdrhvk-tw
		PW FD S P FY WF GG TN C N VDRHV
SEQ ID NO:39	124	ygdevayyfeqdrwnslnngrggpvvqetitrllvevkaaqvlr-d
SEQ ID NO:130	117	lgdkvafywegne-----pgettqityhqlqvqcqfsnvlr-k
SEQ ID NO:131	96	rknklaiewegepvd-----gyptdrklttydlyrevnravaymlqn
		GD VA Y EG D G P IT LLVEV A VLR
SEQ ID NO:39	173	lgkkgdrialnmpnimpqiyyte-aakrlgilytpvfggfsdktlsdri
SEQ ID NO:130	155	qgiqkgdrvaiympmipelvaml-acarigalhsivfagfseslceri
SEQ ID NO:131	141	fgvkkgdkitlylp-mvpelpitmlaawrigaitsvfvsgfsadalaeri
		G KKGDRIAL MP I P T AA R G L VF GFS L RI
SEQ ID NO:39	222	hnagarvvitsdgayrnaqvpykeaytdqal----dkyipvetaqaiva
SEQ ID NO:130	204	ldsscsllittdafyrgeklvnkel-adealqkcqekgfpvrc--civv
SEQ ID NO:131	190	ndsqsrivitadgfwrrgrvvrakev-----
		R VIT DG YR VV KE D AL K PV IV
SEQ ID NO:39	268	qtlatlpltesqrqtiiteveaalageitversdvmrgvgsalaklrld
SEQ ID NO:130	251	khlgrael-----gmgdsts-----
SEQ ID NO:131	216	-----vdaal-----
		L L V AAL G G
SEQ ID NO:39	318	asvqakvrtvlaqalvespprveavvvvrhtg-qeilwnegrdrwshdl
SEQ ID NO:130	266	-----qspikrscpdv-----qiswnqgidlwwhelm
SEQ ID NO:131	221	-----ekatgvesvivlprlgkdvpmtegrdywnklm
		ESPP VE V VV G I WNEGRD W H L
SEQ ID NO:39	367	daalakilanaaraagfdvhsendlnlpddqliralysipcep--vdae
SEQ ID NO:130	294	qea-----gde-----cepewcdae
SEQ ID NO:131	255	q-----gipn-----aylepep--vese
		A P D A I CEP VDAE
SEQ ID NO:39	415	ypmfilytsgstgkpgkviwhvggyvagvvhltlrsvfdaepgdtiyviad
SEQ ID NO:130	309	dplfilytsgstgkpgkvvhtvggymlyvattfkvyfdhaedvfwtad
SEQ ID NO:131	272	hpsfilytsgttgkpgkivhdtggwvavhyatmkwvfdirdddifwtad
		P FI YTSGSTGKPGKV H GGY V T FD D AD
SEQ ID NO:39	465	pgwitgqsymltatmagrltgviaegsplfpsagryasiierygvqifka
SEQ ID NO:130	359	igwitghsyvtgplangatsvlfegiptydpvnrlwsivdkykvtkfyt
SEQ ID NO:131	322	igwvthgsyvvlgpllmgateviyegapdyppqdrwsiierygvttifyt
		GWITG SY A T VI EG P P R SIIERYGV IF

SEQ ID NO:39	515	gvtflktvmsnpqnvdevrlydmhsrlrvatfcaepvspavqqfgmqimtp
SEQ ID NO:130	409	aptairllmkfgd--epvthksraslqvlgtvgepinpeawlwyrvvga
SEQ ID NO:131	372	sptairmfmyge--ewprkhdltlriihsvgepinpeawrwayrvlgn
		T M E VR D SLRV EP P
SEQ ID NO:39	565	q---yi---nsywatehggivwthfygnqdfplrpdahtypwvmgdvw
SEQ ID NO:130	457	qrcpiv---dtfwqtetgghmltplpgat--pmkpgsatfp----ffgva
SEQ ID NO:131	420	e---kvafgstwmmtetggivishapglylvpmpkpgtngpplpgfevdv-
		Q W TE GGIV TH G P P T PLP DV
SEQ ID NO:39	609	vaetdesgttryrvadfddekgeivitapypyltrtlwgdvpgfeaylrge
SEQ ID NO:130	498	pailnesg----eelegeaegylvfkqpwpgimrtvy-----
SEQ ID NO:131	466	---vdengnp---appgvgkylvikpwpvgmlhgiw-----
		A DESG A KG VI P P RT W
SEQ ID NO:39	659	iplrawkgdaerfvktywrrgpngegwgiqgdfaikypdgsftlhgrpdd
SEQ ID NO:130	531	-----gnherfettyfkkfpg---yyvtgdgcqrdqdggywitgridd
SEQ ID NO:131	496	-----gdperyktywsxfpg---mfyagdyaikdkdgyiwlgrade
		GD ERF KTYW R P Y GD AIK DG GR DD
SEQ ID NO:39	709	vinvsghrmgteieegailrdrqitpdsdpvgnclivvgaphrekgltpvaf
SEQ ID NO:130	571	mlnvsghllstaevesalve-----heavaeaavvgphphvkgeclycf
SEQ ID NO:131	536	vikvaghrigtylelesali-----shpavaesavvgvpdaikgevpiaf
		VINVSGRH GT E E A V VVG PH KG P AF
SEQ ID NO:39	759	iqpapgrhltgadrrrldelvrtekavsvpedyie-vsafpetrsgkym
SEQ ID NO:130	615	vtlcdgghtfspkkteelkkqirekigpiatp-dyignapglpktrsgkim
SEQ ID NO:131	580	vvlkqgvapsdelrkelrehvrrtigpiaepaqiff-vtklpktrsgkim
		G R L E VR G P DYI V P TRSGK M
SEQ ID NO:39	808	rxflrnmml-deplgdtttlrnpevleeiaakiaewkrrqrmaeeqqie
SEQ ID NO:130	664	rrvlrkiaqndhdldgmstvadpsvi-----
SEQ ID NO:131	629	rrllkavat-gaplgdvt-----
		RR LR D PLGD TT P V
SEQ ID NO:39	857	ryryfrieyphtasagklavvtvtnppvnaalneraldelntivdhlarr
SEQ ID NO:130	690	-----
SEQ ID NO:131	647	-----
SEQ ID NO:39	907	qdvaaiivftgqgarsfvagadirqlleeihtveeamalpnnaahlafrkie
SEQ ID NO:130	690	-----shl-----
SEQ ID NO:131	647	-----ledetsveeak-----
		LE VEEA HL
SEQ ID NO:39	957	rmnkpciaaingvalggglefamachyrvadvyaeqgqpeinlrllpgyg
SEQ ID NO:130	693	-----
SEQ ID NO:131	658	-----
SEQ ID NO:39	1007	gtqrlprllykrnngtgilralemlggrsvpadealklglidaiatgdq
SEQ ID NO:130	693	-----
SEQ ID NO:131	658	-----raye-----
		RA E
SEQ ID NO:39	1057	dsislacalaraaigadgqliesaaavtqafrrrheqldewrkpdprfadd
SEQ ID NO:130	693	-----fshr-----
SEQ ID NO:131	662	-----
		F HR

SEQ ID NO:39 1107 elrsiihnprieriirqahtvgrdaavhraldairygiingfeaglehea
SEQ ID NO:130 697 -----
SEQ ID NO:131 662 -----

SEQ ID NO:39 1157 klfaeavvdpnggkrgirefldrqsaplptrrplitpegeqllrdqkell
SEQ ID NO:130 697 -----
SEQ ID NO:131 662 -----

SEQ ID NO:39 1207 pvgspffpgvdripkwqyaqavirdpdtgaaahgdpivaekqiivpverp
SEQ ID NO:130 697 -----
SEQ ID NO:131 662 -----

SEQ ID NO:39 1257 ranqaliyvlasevnfnldiwaitgipvsrfdehdrdwhvtgsggigliva
SEQ ID NO:130 697 -----
SEQ ID NO:131 662 -----

SEQ ID NO:39 1307 lgeearregrlkvgdilvaiysggsdllsplmgldpmaadfviqgnndtpdg
SEQ ID NO:130 697 -----
SEQ ID NO:131 662 -----

SEQ ID NO:39 1357 shqqfmlaqapqclpiptdmsieaagsyilnlgtiyralfttlqikagrt
SEQ ID NO:130 697 -----cl-----tiq-----
SEQ ID NO:131 662 -----eika-----
CL T QIKA

SEQ ID NO:39 1407 ifiegaatgtgldaarsaarngrlvigmvssssrastllaagahgainrk
SEQ ID NO:130 702 -----
SEQ ID NO:131 666 -----

SEQ ID NO:39 1457 dpevadcftrvpdpdpawaaewaagqpllamfraqndgrladyvvshage
SEQ ID NO:130 702 -----
SEQ ID NO:131 666 -----

SEQ ID NO:39 1507 tafprsfqllgeprdgthiptltfygatsgyhftflgkpgsasptemlrre
SEQ ID NO:130 702 -----
SEQ ID NO:131 666 -----

SEQ ID NO:39 1557 nlrageavliyygvgsddldvdtggleaieaarqmgarivvvvtvsdaqref
SEQ ID NO:130 702 -----
SEQ ID NO:131 666 -----

SEQ ID NO:39 1607 vlsigfgaalrgvvslaelkrrfgdefewprtmplpnarqdpqggleav
SEQ ID NO:130 702 -----
SEQ ID NO:131 666 -----emart-----
E RT

SEQ ID NO:39 1657 rrfndlvfkplgsavgvflrsadnprgypdliieraahdalavsamlikp
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

SEQ ID NO:39 1707 ftgrivvyfediggrrysffapqiwvrqrrimptafigthlsnayeilr
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

SEQ ID NO:39 1757 indeisaglltitepavvpwdelpeahqamwenrhtaatyvvnhalprlg
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

SEQ ID NO:39 1807 lknrdelyeawtager
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

Figure 41

SEQ ID NO:39	1	midtaplappraprsnpirdrvdweaqraaaladpgafhgaiartvihwy
SEQ ID NO:132	1	-----
SEQ ID NO:133	1	-----
SEQ ID NO:39	51	dpqhhcwifrfnessqrwegldaagapvtvdypadyqpwwqafddseapf
SEQ ID NO:132	1	-----
SEQ ID NO:133	1	-----md----- D
SEQ ID NO:39	101	yrwfsggltfnacfnvdrhvmgygdevayyfgdrwdnslnngrggpvpv
SEQ ID NO:132	1	-----melnn-----
SEQ ID NO:133	3	-----fnnv----- FN V LNN
SEQ ID NO:39	151	qetitrrrllvevvkaagvlrdlglkkgdrialnmpnimpqiyyteaakr
SEQ ID NO:132	6	-----
SEQ ID NO:133	7	-----llnkddgial----- L K D IAL
SEQ ID NO:39	201	lgilytpvfggfsdktlrdrihnagarvitsdgayrnaqvvpvpykeaytd
SEQ ID NO:132	6	-----
SEQ ID NO:133	17	-----
SEQ ID NO:39	251	qaldkiypvetaqaivaqtlatlpitesqrqtiiteveaalageitvers
SEQ ID NO:132	6	-----vileke-----
SEQ ID NO:133	17	----- I E E
SEQ ID NO:39	301	dvmrgvgssalaklrlddasvqakvrtvlaqalvespprveavvvvrhtgq
SEQ ID NO:132	12	-----
SEQ ID NO:133	17	-----
SEQ ID NO:39	351	eilwnegrdrwshdlldaalakilanaraagfdvhsendlnlpddqlir
SEQ ID NO:132	12	-----
SEQ ID NO:133	17	-----iin----- I N
SEQ ID NO:39	401	alyasipcepvdaeypmfliytsgstgkpgkghvhggyvagvvtlrsv
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----
SEQ ID NO:39	451	fdaepgdtiyviadpgwitggsymltatmagrltgviaegsplfpsagry
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----
SEQ ID NO:39	501	aslierygvqifkagvtflktvmsnpqnvdrlydmhslrvatfcaepv
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----

SEQ ID NO:39	551	spavqqfgmqimtpqyinsywaterhggivwthfygnqdfplrpdahypl
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----rpka-----
		RP A
SEQ ID NO:39	601	pwvmgdvwvaetdesgttryrvadfdekgeivitapypyltrtlwgdvpg
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	651	feaylrgeiplrawkgdaerfvktywrrgpngegyiqgdfalkypdgsf
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	701	tlhgrpddvinvsghrmgteeeiegailrdqitpdsfvnciivvgaphre
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	751	kgltpvafiqpapgrhltagadrrrldelvrtekavsvpedyievsafpe
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	801	trsgkymrrflrnmmldeplgdtttlrnpevleeiaakiaewkrrqmae
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	851	eqqileryryfrieypptasagklavvtvtnpp-vnalneraldelnti
SEQ ID NO:132	12	-----gkvavvtinrpkalnalsdtlkemdyv
SEQ ID NO:133	25	-----lnalnyetlkeldsv
		GK AVVT P NALN L EL
SEQ ID NO:39	900	vdhlarrqdvaaivftgqgarsfvagadirqlleeihtve-eamalpnna
SEQ ID NO:132	40	igeiendsevlaviltgageksfvagadisem-kemntiegrkfgilgnk
SEQ ID NO:133	40	ldivendkeikvliitgsgektfvagadiaemsn--mtpl-eakkfslyg
		D V A TG G SFVAGADI E T E EA N
SEQ ID NO:39	949	hlafrkiermnkpciaaingvalggglefamachyrvadvyaeffgqpein
SEQ ID NO:132	89	--vfrllellekpviaavngfalgggceiamsdriassnarffgqpevg
SEQ ID NO:133	87	qkvfrkiemlskpviaavngfalgggcelsmacdriasknakfgqpevg
		FRKIE KP IAA NG ALGGG E AMAC R A A FGQPE
SEQ ID NO:39	999	lrlpgygggtqrlprllykrnngtgllralemlggrsvpadealkgli
SEQ ID NO:132	137	lgitpgfggtqrlsrlv-----gmgmakqliftagnikadealriglv
SEQ ID NO:133	137	lgiipgfsqgtqrlprli-----gtskakeliftgdmnsdeaykigli
		L PG GGTQRLPRL G A E I G ADEALK GLI
SEQ ID NO:39	1049	daiatgdqdsislacalaraaigadgqliesaaavtqafrrheqldewrk
SEQ ID NO:132	180	n-----
SEQ ID NO:133	180	skvv-----
SEQ ID NO:39	1099	pdprfaddelrsiahprieriirqahtvgrdaavhraldairygiihgf
SEQ ID NO:132	181	-----
SEQ ID NO:133	184	-----elsdli-----
		EL I

63/98

SEQ ID NO:39 1749 snayeilrlndeisaglltitepavvpwdelpeahqamwenrhtaatyvv
SEQ ID NO:132 257 -----
SEQ ID NO:133 260 -----

SEQ ID NO:39 1799 nhalpriglknrdelyeawtager
SEQ ID NO:132 257 -----gfknr-----
SEQ ID NO:133 260 -----

G KNR

Figure 42

SEQ ID NO:39	1	midtaplappraprsnpirdrvdweaqraaaladpgafhgaiartvihwy
SEQ ID NO:134	1	-----
SEQ ID NO:135	1	-----
SEQ ID NO:39	51	dpqhhcwirfnessqrwegldaagapvtvdypadyqpwwqafddseapf
SEQ ID NO:134	1	-----maasaap-----
SEQ ID NO:135	1	-----
AA AP		
SEQ ID NO:39	101	yrwfsaggltncacfnevdhrvmmggygdevayyfegdrwdnslnngrggppv
SEQ ID NO:134	8	-----
SEQ ID NO:135	1	-----
SEQ ID NO:39	151	qetitrrrllvevvkaaqlrdlglkkgdrialnmpnimpqiyyteaakr
SEQ ID NO:134	8	-----
SEQ ID NO:135	1	-----
SEQ ID NO:39	201	lgilytpvfggfsdktlsdrihnagarvvitsdgayrnaqvpykeaytd
SEQ ID NO:134	8	-----awtg
SEQ ID NO:135	1	-----
A T		
SEQ ID NO:39	251	qaldkyipvetaqaivaqtlatlpltesqrqtiiteveaalageitvers
SEQ ID NO:134	12	q-----taeak
SEQ ID NO:135	1	-----mtlqtlettalkd-----
Q QTL T L T E		
SEQ ID NO:39	301	dvmrgvgsalaklrldasvqakvrtvlaqalvespprveavvvvrttgq
SEQ ID NO:134	18	d-----
SEQ ID NO:135	14	-----
D		
SEQ ID NO:39	351	eilwnegrdrwshdlldaalakilanaraagfdvhsendlilnpddqlir
SEQ ID NO:134	19	-----
SEQ ID NO:135	14	-----
SEQ ID NO:39	401	alyasipcepvdaeypmfiiytsgstgkpkgvihvhggyvagvvhtlrvs
SEQ ID NO:134	19	-----
SEQ ID NO:135	14	-----
SEQ ID NO:39	451	fdaepgdtiyviadpgwitgqsymltatmagrltgviaegsplfpsagry
SEQ ID NO:134	19	-----
SEQ ID NO:135	14	-----
SEQ ID NO:39	501	aslierygvqifkagvtflktvmsnpqnvdevrlydmhslrvatfcaepv
SEQ ID NO:134	19	-----lyel-----
SEQ ID NO:135	14	-----lyei-----
LY		

SEQ ID NO:39	551	spavqqfgmqimtpqyinsywaterhggivwthfygnqdfplrpdahyp1
SEQ ID NO:134	23	-----
SEQ ID NO:135	18	-----
SEQ ID NO:39	601	pwvmgdvwvaetdesgttryrvadfdekgeivitapypyltrtlwgdvpg
SEQ ID NO:134	23	-----
SEQ ID NO:135	18	-----
SEQ ID NO:39	651	feaylrgeiplrawkgdaerfvktywzrgpngegyiqgdfaikypdgsf
SEQ ID NO:134	23	-----geip-----
SEQ ID NO:135	18	-----geip-----
		GEIP
SEQ ID NO:39	701	tlhgrpddvinvsghrmgteieiegallrdrqitpdspgncivvgaphre
SEQ ID NO:134	27	-----
SEQ ID NO:135	22	-----
SEQ ID NO:39	751	kgltpvafiqpapgrhltdgarrzrldeivrtkgavsvpedyievsafpe
SEQ ID NO:134	27	-----
SEQ ID NO:135	22	-----pafhv-----pk
		P H P
SEQ ID NO:39	801	trsgkymrrflrnmmldeplgdtttlrnpevleeiaakiaewkrrqmae
SEQ ID NO:134	27	-----plg-----hvpakmyawairr-----
SEQ ID NO:135	29	t-----nyawsirk-----
		T PLG AK W R
SEQ ID NO:39	851	eqqileryryfrieypptasagklavvtvtnppvnalneraldeltiv
SEQ ID NO:134	43	-----erh-----
SEQ ID NO:135	38	-----
		ER
SEQ ID NO:39	901	dhlarrqdvaaivftgqgarsfvagadirqlleeihtveeamalpnahl
SEQ ID NO:134	46	-----
SEQ ID NO:135	38	-----
SEQ ID NO:39	951	afrkiermnkpciaaingvalggglefamachyrvadvyaefggpseinlr
SEQ ID NO:134	46	-----gppe-----
SEQ ID NO:135	38	-----erhgap-----
		ER KP G PE
SEQ ID NO:39	1001	llpgygggtqrlprllykrnngtgllralemilggrsvpadealkglida
SEQ ID NO:134	50	-----
SEQ ID NO:135	44	-----
SEQ ID NO:39	1051	iatgdqdsllslacalaraaigadgqliesaaavtqafrrheqldewrkpd
SEQ ID NO:134	50	-----
SEQ ID NO:135	44	-----tqamq-----
		TQA
SEQ ID NO:39	1101	prfaddelrsiihprrieriirqahtvgrdaavhraldairygiingfea
SEQ ID NO:134	50	-----qsh-----
SEQ ID NO:135	49	-----
		Q H

```

SEQ ID NO:39 1151 gleheaklf aeavvdpnggkrgirefldrqsaplptrrplitpeqeqlir
SEQ ID NO:134 53 -----
SEQ ID NO:135 49 -----

SEQ ID NO:39 1201 dqkellpvgs pffpgvdripkwqyaqavirdpdtgaaahgdpivaekqii
SEQ ID NO:134 53 -glevlpv-----wei-----gd-----
SEQ ID NO:135 49 -----vevvptweige-----
                        Q E LPV          V P W          GD

SEQ ID NO:39 1251 vpverpranqaliyv lasevnfndiwaitgipvsrfdehdrdwhtvgsgg
SEQ ID NO:134 65 -----devlvymaagvnyngvwaglgepis pfdvhkgeyhiagsda
SEQ ID NO:135 60 -----devlvymaagvnyngvwaalgepis pldghkqpfhiagsda
                        L Y V A V N N W A G P S F D H H G S

SEQ ID NO:39 1301 igli valgeearregrlkv gdlvaiysggsdllsp-lmgl dpm-aadf v-
SEQ ID NO:134 107 sgiwkv gakvk---rwkv gdevivhcnqddg ddeecnggdpm-fsptqr
SEQ ID NO:135 102 sgiwkv gakvk---rwkl gdevvihcnqddg ddeecnggdpmfsssq r-
                        G G R K V G D V I Q D G D P M

SEQ ID NO:39 1348 i qgndtpdgshqqfmla qapqc lpiptdmsieaagsyi lnltiyral f-
SEQ ID NO:134 153 iwgyetp dgsfaqfcrvqsrqlmarpkhltweeaacytltlatayrmlfg
SEQ ID NO:135 148 iwgyetp dgsfaqfcrvqsrqlprpkhltweesacytltlatayrmlfg
                        I G T P D G S Q F Q Q L P P E A Y L L T Y R L F

SEQ ID NO:39 1397 -tti qikagrt ifiegaatgtgl daarsaarn glrvigmvsssrastll
SEQ ID NO:134 203 haptv rpgqnvliw gasgglgvfgvqlcaasganaiavisdeskrdyvm
SEQ ID NO:135 198 hkphelkpgqnvliw gasgglgvfatqlaavaganaigvssedkrefvl
                        K G I G A G G A A A G I G V S S S L

SEQ ID NO:39 1446 aagah gainrkdpevadcftrvpedpsawaaweaaagqpllamfra qndgr
SEQ ID NO:134 253 slgakgv inrkd---fdc---w-----
SEQ ID NO:135 248 smgakavlnrge---fncw gqlpk-----
                        G A G I N R K D D C P

SEQ ID NO:39 1496 ladyv vshagetafprsfql lgeprdg hiptltfygatsgyhftflgkpg
SEQ ID NO:134 269 -----gqlptv-----
SEQ ID NO:135 269 -----vngpef-----
                        G P T G F

SEQ ID NO:39 1546 sasptemlrranlrageavliyygvg sddlvdtgg lealeaarqmgariv
SEQ ID NO:134 275 -----
SEQ ID NO:135 275 -----

SEQ ID NO:39 1596 vvtvsdaqrefvls lsgfgaalrgvvs laelkrrf gdefewprtmpplpna
SEQ ID NO:134 275 -----ns
SEQ ID NO:135 275 ---ndymke-----srkfgkai-wqit-----
                        D E R F G W T N

SEQ ID NO:39 1646 rqp dpglkeavrrfndlvfkplgsavgvflrsadnprgypdliieraahd
SEQ ID NO:134 277 peyntwlkea-rkfgkaiwditgk gndv-----divfehpg ea
SEQ ID NO:135 293 ----gnkdv-----dmvfehpg eq
                        G L K E A R F G V D E

SEQ ID NO:39 1696 alavsa mlikpftgriv yfediggrrysfapqliwvrq rriymptaqifg
SEQ ID NO:134 314 tfpvstlvakr-ggmivfcagttgfnitfdaryvwmrqkriq-----g
SEQ ID NO:135 308 tfpvsfvlvkr-ggmvvicagttgfnltmdarflwmrqkrvq-----g
                        V S L K G I V G F A W R Q R I G

```

SEQ ID NO:39 1746 thlsnayeilrlndeisaglltitepavvpwdeipeahqamwenrhta
SEQ ID NO:134 356 shfahlkqasaanqfvmddrvdpcmsevpwdkipaahkmwknqhppgn
SEQ ID NO:135 350 shfanlmqasaanqlvidrrvdpcisevpwdqipaahekmlanqhlpgn
H N N V PWD P AH MW N H

SEQ ID NO:39 1796 yvvnhalprlgknrdelyeawtager
SEQ ID NO:134 406 mavlvnstraglrtvedvieagplkam
SEQ ID NO:135 400 mavlvcaqrpglrtfeevqelsgap--
V R GL E EA A

Figure 43

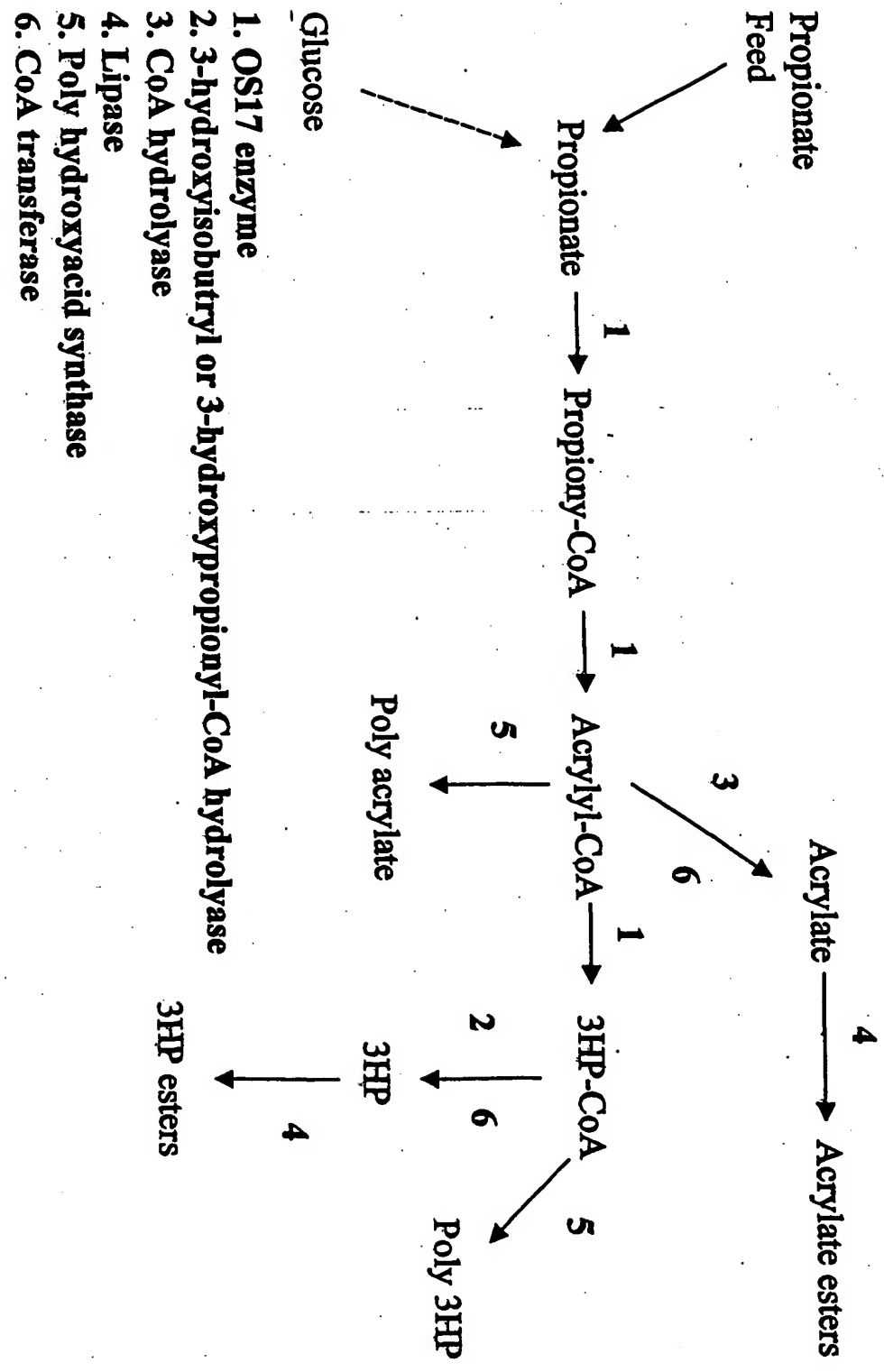


Figure 44

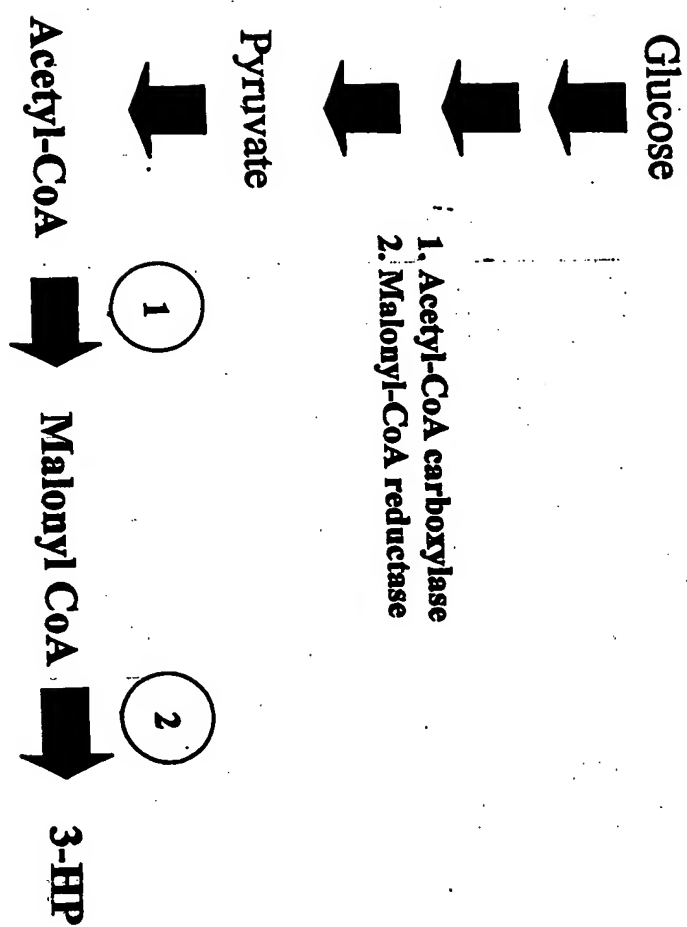
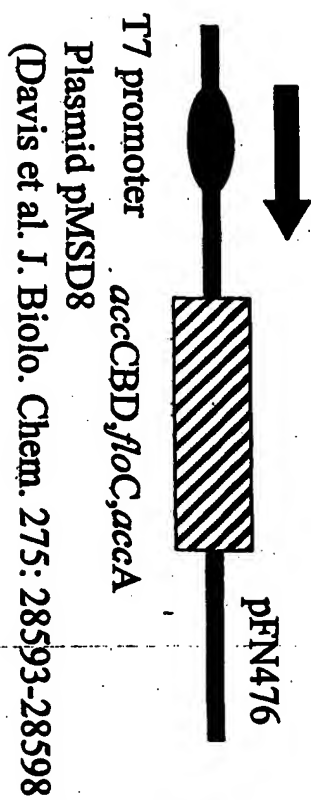


Figure 45

Acetyl-CoA carboxylase constructs



Malonyl-CoA reductase constructs

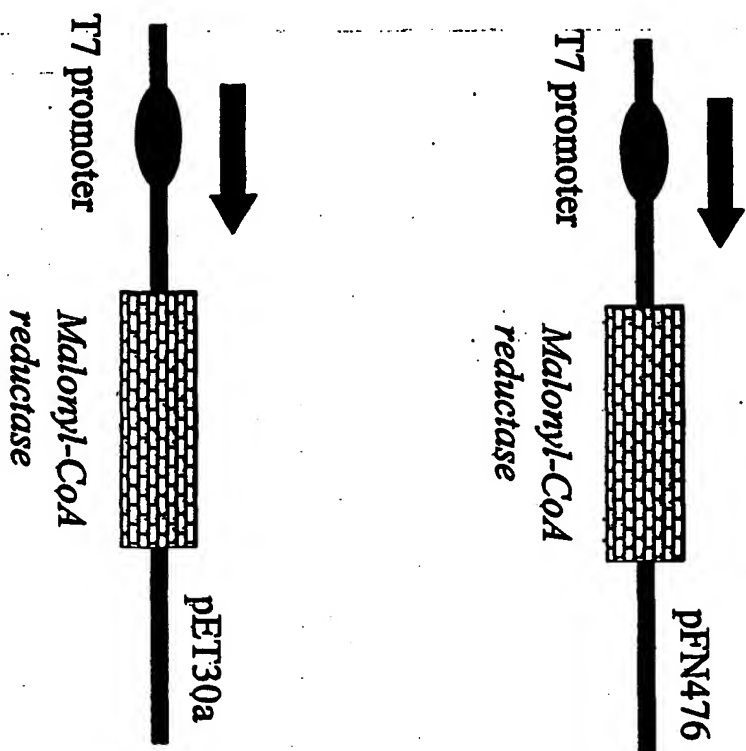


Figure 46

Relative Detector Response

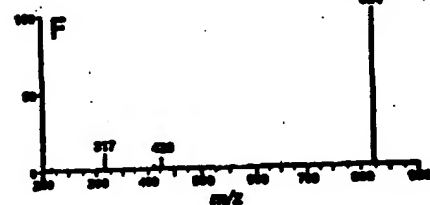
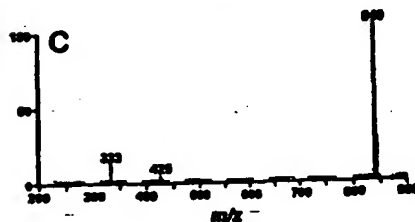
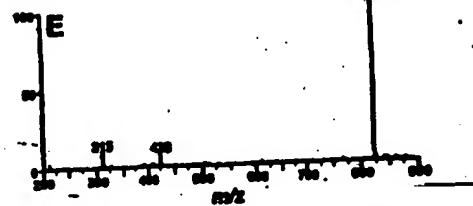
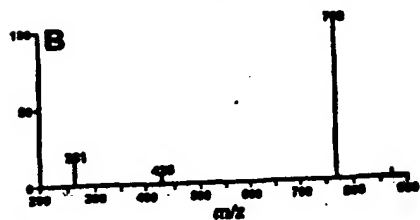
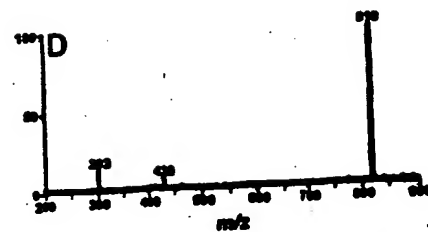
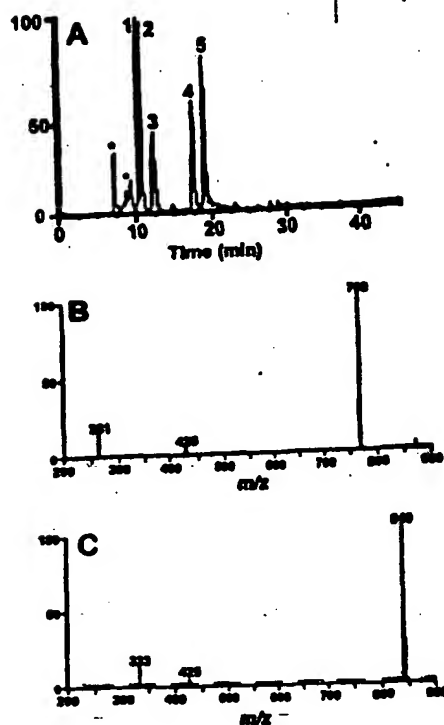


Figure 47

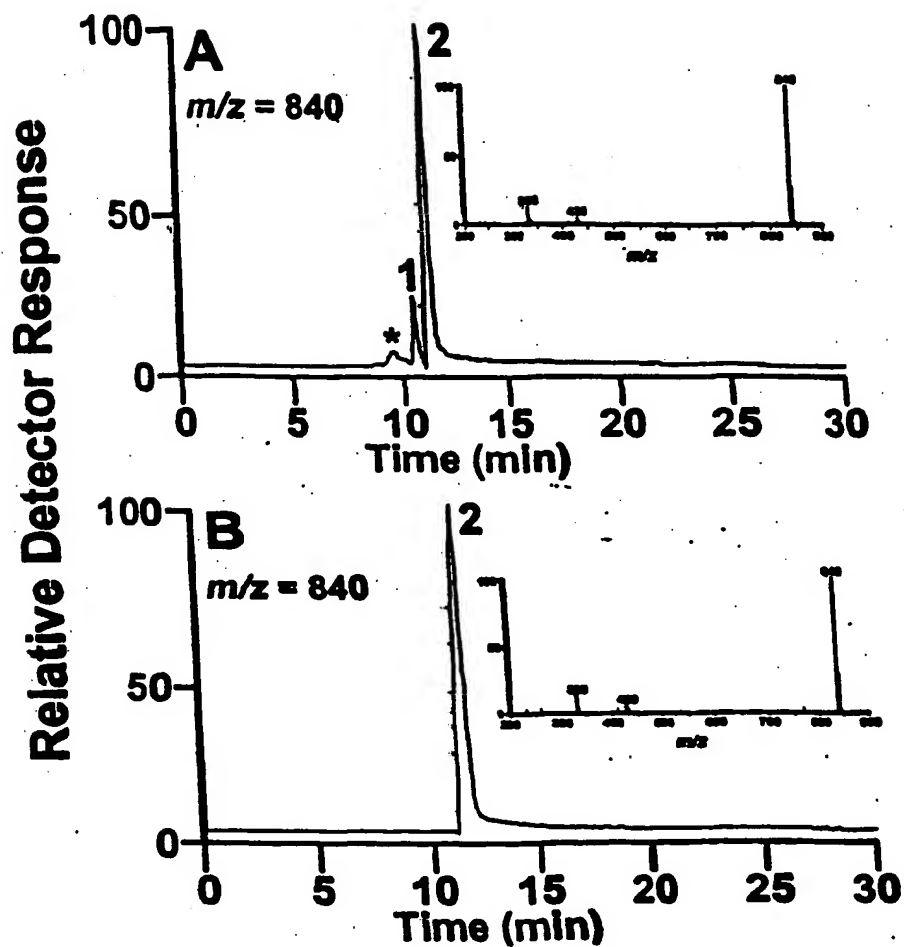


Figure 48

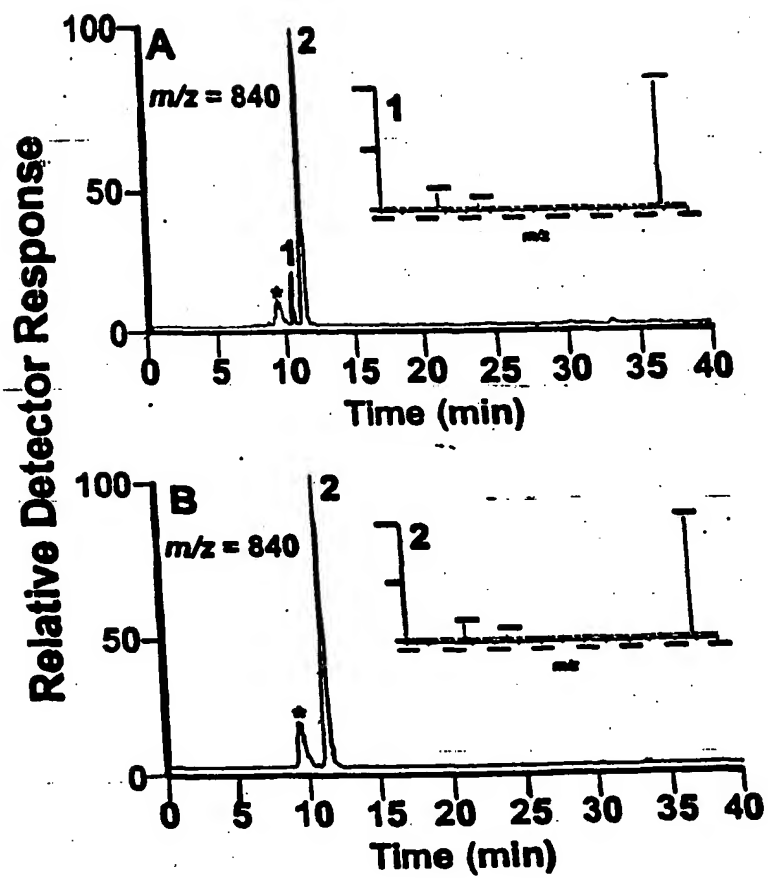


Figure 49

ATGGCGACGGGGGAGTCCATGAGCGGAACAGGACGACTGGCAGGAAAGATTGCGTTAATT
ACCGGTGGCGCGCGCAATATCGGCAGTGAATTGACACGTCGCTTTCTCGCAGAGGGAGCG
ACGGTCATTATTAGTGGACGGAATCGGGCGAAGTTGACCGCACTGGCCGAACGGATGCAG
GCAGAGGCAGGAGTGCCGGCAAAGCGCATCGATCTGGAAGTCATGGATGGGAGTGATCCG
GTCGCGGTACGTGCCGGTATCGAAGCGATTGTGGCCCGTCACGGCCAGATCGACATTCTG
GTCAACAATGCAGGAAGTGCGGGTCCCCAGGGTCGTCTGGCCGAGATTCCACTCACTGAA
GCTGAATTAGGGCCCTGGCGCCGAAGAGACGCTTCATGCCAGCATGGCCAATTTACTTGGT
ATGGGATGGCATCTGATGCGTATTGCGGCACCTCATATGCCGGTAGGAAGTGCGGTCATC
AATGTCTCGACCATCTTTTACGGGCTGAGTACTACGGGCGGATTCCGTATGTCACCCCT
AAAGCTGCTCTTAATGCTCTATCTCAACTTGCTGCGCGTGAGTTAGGTGCACGTGGCATC
CGCGTTAATACGATCTTTCCCGGCCCGATTGAAAGTGATCGCATCCGTACAGTGTTCCAG
CGTATGGATCAGCTCAAGGGGGCGGCCGAAGGCGACACAGCGCACCATTTTTTGAACACC
ATGCGATTGTGTCGTGCCAACGACCAGGGCGCGCTTGAACGTCGGTTCCCTCGGTGCGT
GATGTGGCAGACGCGCGCTGTCTTCTGGCCAGTGCCGAATCCGCGCTCTCTCCGGTGAG
ACGATTGAGGTTACGCACGGAATGGAGTTGCCGGCTGCAGTGAGACCAGCCTGCTGGCC
CGTACTGATCTGCGCACGATTGATGCCAGTGGCCGACGACGCTCATCTGCGCCGGCGAC
CAGATTGAAGAGGTGATGGCGCTCACC GGATGTTGCGTACCTGTGGGAGTGAAGTGATC
ATCGGCTTCCGTTCCGGCTGCGGCGCTGGCCAGTTTCGAGCAGGCAGTCAATGAGAGTCGG
CGGCTGGCGGGCGCAGACTTTACGCCTCCCATTGCCTTGCCACTCGATCCAGGCGATCCG
GCAACAATTGACGCTGTCTTCGATTGGGCGCGGAGAAATACCGGCGGGATTTCATGCAGCG
GTGATTCTGCCTGCTACCAGTCACGAACCGGCACCGTGCCTGATTGAGGTTGATGATGAG
CGGGTGCTGAATTTTCTGGCCGATGAAATCACEGGGACAATTGTGATTGCCAGTCGCCTG
GCCCGTTACTGGCAGTCGCAACGGCTTACCCCCGGCGACGTGCGCGTGGGCGCGGTGTC
ATTTTTCTCTCGAACGGTGCCGATCAAAATGGGAATGTTTACGGACGCATTCAAAGTGCC
GCTATCGGTCAGCTCATTCGTGTGTGGCGTCACGAGGCTGAACTTGACTATCAGCGTGCC
AGCGCCGCGCGTGATCATGTGCTGCCCGCGGTATGGGCCAATCAGATTGTGCGCTTCGCT
AACC GCAGCCTTGAAGGGTTAGAATTTGCCTGTGCCTGGACAGCTCAATTGCTCCATAGT
CAACGCCATATCAATGAGATTACCCTCAACATCCCTGCCAACATTAGCGCCACCACGGC
GCACGCAGTGCATCGGTGGATGGGCGGAAAGCCTGATCGGGTTGCATTTGGGGAAAGTT
GCCTTGATTACCGGTGGCAGCGCCGGTATTGGTGGGCAGATCGGGCGCCTCCTGGCTTTG
AGTGGCGCGCGCGTGATGCTGGCAGCCCGTGATCGGCATAAGCTCGAACAGATGCAGGCG
ATGATCCAATCTGAGCTGGCTGAGGTGGGGTATACCGATGTCGAAGATCGCGTCCACATT
GCACCGGGCTGCGATGTGAGTAGCGAAGCGCAGCTTGCGGATCTTGTTGAACGTACCCTG
TCAGCTTTTGGCACCGTCGATTATCTGATCAACAACGCCGGGATCGCCGGTGTCGAAGAG
ATGGTTATCGATATGCCAGTTGAGGGATGGCGCCATACCCTCTTCGCCAATCTGATCAGC
AACTACTCGTTGATGCGCAAACTGGCGCCGTTGATGAAAAACAGGGTAGCGGTTACATC
CTTAACGTCTCATCATACTTTGGCGGTGAAAAAGATCGGGCCATTCCCTACCCCAACCGT
GCCGATTACGCCGTCTCGAAGGCTGGTCAGCGGGCAATGGCCGAAGTCTTTGCGCGCTTC
CTTGGCCCGGAGATACAGATCAATGCCATTGCGCCGGTCCGGTTCGAAGGTGATCGCTTG
CGCGGTACCGGTGAACGTCCCGGCCTCTTTGCCCGTCGGGCGCGGCTGATTTTGGAGAAC
AAGCGGCTGAATGAGCTTCACGCTGCTCTTATCGCGGCTGCGCGCACCGATGAGCGATCT
ATGCACGAACTGGTTGAACTGCTCTTACCCAATGATGTGGCGCACTAGAGCAGAATCCC
GCAGCACCTACCGCGTTGCGTGAACTGGCAGCAGCTTTTCGAGCGGAAGGCGATCCGGCG
GCATCATCAAGCAGTGCGCTGCTGAACCGTTCAATTGCCGCTAAATTGCTGGCTCGTTTG
CATAATGGTGGCTATGTGTTGCCTGCCGACATCTTTGCAAACCTGCCAAACCCGCCCGAT
CCCTTCTTACCCGAGCCAGATTGATGCGGAGGCTCGCAAGGTTTCGTGAEGGCATCATG
GGGATGCTCTACCTGCAAGGATGCGGACTGAGTTTGATGTGCAATGGCCACCGTCTAT
TACCTTGCCGACCGCAATGTCA GTGGTGAGACATTCCACCCATCAGGTGGTTTGGCTTAC

GAACGCACCCCTACCGGTGGCGAACTCTTCGGCTTGCCCTCACGGGAAGGGCTGGCGGAG
CTGGTCGGAAGCACGGTCTATCTGATAGGTGAACATCTGACTGAACACCTTAACCTGCTT
GCCCCGTGCGTACCTCGAACGTTACGGGGCACGTCAGGTAGTGATGATTGTTGAGACAGAA
ACCGGGGCAGAGACAATGCGTCGCTTGCTCCACGATCACGTCGAGGCTGGTCGGCTGATG
ACTATTGTGGCCGGTGATCAGATCGAAGCCGCTATCGACCAGGCTATCACTCGCTACGGT
CGCCCAGGGCCGGTCGTCTGTACCCCTTCCGGCCACTGCCGACGGTACCACTGGTCGGG
CGTAAAGACAGTGACTGGAGCACAGTGTTGAGTGAGGCTGAATTTGCCGAGTTGTGCGAA
CACCAGCTCACCCACCATTTCCGGGTAGCGCGCAAGATTGCCCTGAGTGATGGTGCCAGT
CTCGCGCTGGTCACTCCCGAACTACGGCTACCTCAACTACCGAGCAATTTGCTCTGGCT
AACTTCATCAAAACGACCCTTCACGCTTTTACGGCTACGATTGGTGTCGAGAGCGAAAGA
ACTGCTCAGCGCATTCTGATCAATCAAGTCGATCTGACCCGGCGTGCGCGTGCCGAAGAG
CCGCGTGATCCGCACGAGCGTCAACAAGAACTGGAACGTTTTATCGAGGCAGTCTTGCTG
GTCCTGCACCACTCCCGCTGAAGCCGATACCCGTTACGCCGGGCGGATTTCATCGCGGA
CGGGCGATTACCGTGTA (SEQ ID NO:140)

Figure 50

MATGESMSGTGRLAGKIALITGGAGNIGSELTRRFLAEGATVIISGRNRAKLTALAERMQ
AEAGVPAKRIDLEVMDGSDPVAVRAGIEAIVARHGQIDILVNNAGSAGAQRRLAEIPLTE
AELGPGAETLHASIANLLGMGWHLMRIAPHMPVGSVINVESTIFSRAEYYGRIPYVTP
KAALNALSQLAARELGARGIRVNTIFPGPIESDRITVFQRMQDLKGRPEGDTAHHFLNT
MRLCRANDQGALERRFPSVGDVADA AVFLASAESAALSGETIEVTHGMELPACSETSLA
RTDLRTIDASGRTTLICAGDQIEEVMALTMGLRTCGSEVIIGFRSAAALAQFEQAVNESR
RLAGADFTPPIALPLDPRDPATIDAVFDWAGENTGGIHAAVILPATSHEPAPCVIEVDDE
RVLNFLADEITGTIVIASRLARYWQSQRITPGARARGPRVIFLSNGADQNGNVYGRIQSA
AIGQLIRVWRHEAELDYQRASAAGDHVLPVWVWQIVRFANRSLEGLEFACAWTAQLLHS
QRHINEITLNI PANISATTGARSASVGWAE SLIGLHLGKVALITGGSAGIGGQIGRLAL
SGARVMLAARDRHKLEQMAMIQSELAEVGYTDVEDRVHIA PGCDVSSEAQLADLVERTL
SAFGTVDYLINNAGIAGVEEMVIDMPVEGWRLTLFANLISNYSLMRKLAPLMKKQSGYI
LNVSSYFGGEKDAAIPYPNRADYAVSKAGQRAMAEVFARFLGPEIQINAIAPGPVEGDRL
RGTGERPGLFARRARLILENKRINELHAALIAAARTDERSMHVELLLPNDVAALQNP
AAPTALRELARRFRSEGDPAASSSSALNRSIAAKLLARLHNGGYVLPADIFANLPNPPD
PFFTRAQIDREARKVRD GIMGLYLQRMPT EFDVAMATVYYLADRVNVSGETFHPSGGLRY
ERTPTGGELFGLPSPERLAELVGSTVYLIGEHLTEHLNLLARAYLERYGARQVVMIVETE
TGAETMRRLLDHVEAGRLMTIVAGDQIEAAIDQAITRYGRPGPVVCTPFRPLPTVPLVG
RKDSDWSTVLSEAEFAELCEHQLTHHFRVARKIALSDGASLALVTPETTATSTTEQFALA
NFIKTTLHAFTATIGVESERTAQRILINQVDLTRRARAEEPRDPHERQOELERFIEAVLL
VTAPLPPEADTRYAGRIHRGRAITV (SEQ ID NO:141)

Figure 51

TCTTTCTGGCCAGTGCCGAATCCGCCGCTCTCTCCGGTGAGACGATTGAGGTTACGCAGG
GAATGGAGTTGCCGGCCTGCAGTGAGACCAGCCTGCTGGCCGGTACTGATCTGCGCACGA
TTGATGCCAGTGGCCGCACGACGCTCATCTGCGCCGGCGACCAGATTGAAGAGGTGATGG
CGCTCACCGGTATGTTGCGTACCTGTGGGAGTGAAGTGATCATCGGCTTCCGTTCCGGCTG
CGGCGCTGGCCCAGTTCGAGCAGGCAGTCAATGAGAGTCGGGGGCTGGCCGGGGCAGACT
TTACGCCTCCCATTCGCTTGCCACTCGATCCACGCG (SEQ ID NO:142)

Figure 52

SEQ ID NO:141	1	matgesmsqgrlagkialitggagnigseltrrflaegatviisgrnra
SEQ ID NO:143	1	-----mfankvvltvgssgigaatveafvkegasvafvgrnqa
SEQ ID NO:144	1	-----mrlegkvclitgaasgigkattllfaqegatviagdiske
SEQ ID NO:145	1	-----
SEQ ID NO:146	1	-----mekf-----
SEQ ID NO:147	1	-----mrllhkrtlvttggsdgiglaiaaeflsegadvliivrdaa
SEQ ID NO:141	51	ktalaermqa--e-agvpakridlevmdgsdpvavragieaivarhgqi
SEQ ID NO:143	40	klkevesrcqg--hganilaikadv-----skdeeakiivqqtvdkfgkl
SEQ ID NO:144	41	nldslvk--ea--e--glp-----gkv
SEQ ID NO:145	1	-----
SEQ ID NO:146	5	-----
SEQ ID NO:147	41	kleaarqklaalgq--aga---vetssadlatslgvatvveqvketgrpl
SEQ ID NO:141	98	dilvnnagsagaqrllaeiplteaelpgaeetlhasianllgmghlrmr
SEQ ID NO:143	83	dvlvnnagii---rfasv--leptliqtfdetmntnlrpvv---lits
SEQ ID NO:144	57	d-----
SEQ ID NO:145	1	-----
SEQ ID NO:146	5	-----
SEQ ID NO:147	86	dipinnagvadl-----vpfesv-----seaqfghsfalnvaaffltq
SEQ ID NO:141	148	laaphm-pvgsavinvtifsr-aeyygrip--yvtpkaaalnalsqlaar
SEQ ID NO:143	123	laiphliatkgisinvssilstivripgims--ysvskaaandhftklaal
SEQ ID NO:144	58	-----p--yv-----lnv-----
SEQ ID NO:145	1	-----
SEQ ID NO:146	5	---php-p-----
SEQ ID NO:147	125	gllphf-gagasiinissyfar-kmipkrpssvyslskgalnsitrslaf
SEQ ID NO:141	194	elgargirvntifpgpiesdrirtvfqrmddqlkgripegdtahhfintmrl
SEQ ID NO:143	171	elapsgvrvnsvnpgpv-----
SEQ ID NO:144	64	-----tdr-----
SEQ ID NO:145	1	-----mnpmdrqtgqepqh-----
SEQ ID NO:146	9	-----
SEQ ID NO:147	173	elgprgirvnaiapgtvdt-----
SEQ ID NO:141	244	crandqgalerrfsvgdvadaavflasaesaalsgetievthgmelpac
SEQ ID NO:143	188	-----ltdia-----
SEQ ID NO:144	67	-----
SEQ ID NO:145	16	-----
SEQ ID NO:146	9	-----fpr-----
SEQ ID NO:147	192	-----amrr-----
SEQ ID NO:141	294	setsllartdlrtidasgrttlicagdqieevmaltgmirtcgseviigf
SEQ ID NO:143	193	-----
SEQ ID NO:144	67	-----dqikev-----
SEQ ID NO:145	16	-----
SEQ ID NO:146	12	-----qtgem-----
SEQ ID NO:147	196	-----ktvd-----
SEQ ID NO:141	344	rsaaalaqfeqavnesrrlagadftppialpldprdpavidavfdwagen
SEQ ID NO:143	193	-----agsgfspdli-----ed
SEQ ID NO:144	73	-----
SEQ ID NO:145	16	-----qdrqpgieskmp-----
SEQ ID NO:146	17	-----pgttdrm-----
SEQ ID NO:147	200	-----

SEQ ID NO:141	394	tggihaavilpatshepapcvievddervlnfladeitgtiviasrlary
SEQ ID NO:143	205	tg-----ahtp-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	29	-----lp-----
SEQ ID NO:146	24	-----qplp-----
SEQ ID NO:147	200	-----
SEQ ID NO:141	444	wqsqrlltpgarargprviflsngadqngnvvgriqsaaigqlirvwrhea
SEQ ID NO:143	211	-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	31	-----lsededyrgs--gklk-----
SEQ ID NO:146	28	-----dhg-----
SEQ ID NO:147	200	-----
SEQ ID NO:141	494	eldyqrasaagdhvlpvwanqivrfanrsleglefacaawtaqlhsqrh
SEQ ID NO:143	211	-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	45	-----
SEQ ID NO:146	31	ensyqgsgrlkd-----
SEQ ID NO:147	200	-----
SEQ ID NO:141	544	ineitlnipanisattgarsasvsgwaesliglhlgkvalitggsagiggg
SEQ ID NO:143	211	-----lgkaa-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	45	-----gkvailitggsdgigra
SEQ ID NO:146	43	-----kraitggsdgigra
SEQ ID NO:147	200	-----nlpa-----
SEQ ID NO:141	594	igrllalsgarvmlaardrhk-leqmqamiqselaevgytdvedrvhiap
SEQ ID NO:143	216	-----qse-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	61	aaiafakegadisilyldehsdaestrkrieke-----nvrcllip
SEQ ID NO:146	58	vaiaayaregadvlisylsehd-----damatkalve-----eagrkvlaa
SEQ ID NO:147	204	-----
SEQ ID NO:141	643	gcdvsseaqladivertlsafgtvdylinnagiagveemvidmpvegwrh
SEQ ID NO:143	219	-----eiadmi-----
SEQ ID NO:144	73	-----vekvvqkygridvlvnnagitrdallvrmkeedwda
SEQ ID NO:145	102	g-dvgdenhceqavqqtvdhfgkldilvnnaaeqhpqdsilnisteqlek
SEQ ID NO:146	99	g-diqssdhcrrivetavrelggidilvnnaahqatfkniedisdeewel
SEQ ID NO:147	204	-----eakaekayvers-----
SEQ ID NO:141	693	tlfanlisnyslmrklaplmmkkgsgyilnvssyfggekdaaipypnrad
SEQ ID NO:143	225	-----
SEQ ID NO:144	109	vinvnlkgvfnvtqmvvpymikqrngsivnvssvvg-----iygnpgqtn
SEQ ID NO:145	151	tfrtnifsmfhttkkalphl--qegcalinttsitayegdtal-----id
SEQ ID NO:146	148	tfrvnmhamfylvtkaaavphmkk-gsa-iintasi-----nadvpnpilla
SEQ ID NO:147	217	-----
SEQ ID NO:141	743	yavskagqramaevfarfl-gpe-iqinalapgpvegdrllrgtgerpglf
SEQ ID NO:143	225	-----
SEQ ID NO:144	154	yaaskagvigmtktwakelagrnlirvnavapgfi-----
SEQ ID NO:145	194	ysstkgaivsftrsmaksl-adkgirvnavapgpi-----
SEQ ID NO:146	191	yattkgaihfnfsaglaqml-aergirvnavapgpi-----
SEQ ID NO:147	217	ypigrigr-----

SEQ ID NO:141	791	arrarlilenkrinelhaaliaaartdersmhelveilllpndvaaaleqnp
SEQ ID NO:143	225	-----
SEQ ID NO:144	189	-----
SEQ ID NO:145	228	-----wtp
SEQ ID NO:146	225	-----wtp lipstmpedtva-dfgk
SEQ ID NO:147	225	-----pddlagm---
SEQ ID NO:141	841	aaptalrelarrfrsegdpaassssallnrsiaakllarlhnggyvlpad
SEQ ID NO:143	225	-----
SEQ ID NO:144	189	-----
SEQ ID NO:145	231	lipatfpe-----
SEQ ID NO:146	244	qvp-----mkrgpgpvelasa-----yvmld
SEQ ID NO:147	232	-----
SEQ ID NO:141	891	ifanlpnppdpfftraqidrearkvrdgimgmlylqrmptefdvamatvy
SEQ ID NO:143	225	-----vy
SEQ ID NO:144	189	-----
SEQ ID NO:145	239	-----ekvkq-----
SEQ ID NO:146	266	pmssy-----
SEQ ID NO:147	232	-----av
SEQ ID NO:141	941	yladrnvsgetfhpsgglyertyptggelfglpsperlaelvgstvyliq
SEQ ID NO:143	227	lasdk-----aksvtgscyl---
SEQ ID NO:144	189	-----tpmteklpekareta---
SEQ ID NO:145	244	-----hgldtp-----
SEQ ID NO:146	271	-----vsgatiavtgg-----
SEQ ID NO:147	234	yla-----sdeaawtsggi-----
SEQ ID NO:141	991	ehltehlnllaraylerygarqvmivetetgaetmrrllhdhveagrlm
SEQ ID NO:143	242	-----
SEQ ID NO:144	204	-----lsriplgrfgkpe-----evaqvi
SEQ ID NO:145	250	-----
SEQ ID NO:146	282	-----
SEQ ID NO:147	248	-----
SEQ ID NO:141	1041	tivagdqleaaaidqaitrygrpgpvvctpfprplptvplvgrkdsdwstvl
SEQ ID NO:143	242	-----
SEQ ID NO:144	223	lflasdessyvtgqvi---gidgglyi-----
SEQ ID NO:145	250	-----mgrpgqp-----
SEQ ID NO:146	282	-----kpfl-----
SEQ ID NO:147	248	-----favdggyt-----
SEQ ID NO:141	1091	seae faelcehqithhfrvarkialsdgaslalvtpettatstteqfala
SEQ ID NO:143	242	-----mdnglalq-----
SEQ ID NO:144	247	-----
SEQ ID NO:145	258	-----eha-----gayvllasdes-----
SEQ ID NO:146	286	-----
SEQ ID NO:147	256	-----
SEQ ID NO:141	1141	nfikttlhaftatigvesertaqrilingvdltrraraeprdpheqqe
SEQ ID NO:143	250	-----
SEQ ID NO:144	247	-----
SEQ ID NO:145	272	-----symtggtihvn-----
SEQ ID NO:146	286	-----
SEQ ID NO:147	256	-----

SEQ ID NO:141	1191	lerfieavllvtaplppeadtryagrihrgraitv
SEQ ID NO:143	250	-----
SEQ ID NO:144	247	-----
SEQ ID NO:145	283	-----ggrfist
SEQ ID NO:146	286	-----
SEQ ID NO:147	256	-----ag-----

Figure 53

SEQ ID NO:140	1	atggcgacggggagtcctatgagcggaacaggacgactggcaggaaagat
SEQ ID NO:148	1	-----atga-----gacttctgcacaagcg
SEQ ID NO:149	1	-----atg-----ttcgaaataaagt
SEQ ID NO:150	1	-----atgaggcttgagggaag--
SEQ ID NO:151	1	-----atggaaa--
SEQ ID NO:152	1	-----
SEQ ID NO:140	51	tgcgt-taattaccggtggcgccggcaatatcggcagtgaattgacacgt
SEQ ID NO:148	21	cacgc-tggtgaccggcggtc-----
SEQ ID NO:149	18	ggtac-tagtaacagggtgtagctccggtatcggc-----
SEQ ID NO:150	20	tgtgtctgatcacagg---ggctgcaagcgggatagggaag-gccacca
SEQ ID NO:151	8	-----aattccgca-----ccct
SEQ ID NO:152	1	-----
SEQ ID NO:140	100	cgctt--tctcgagagggagcgacgggtcattattagtgacggaatcgg
SEQ ID NO:148	42	-----ggacggtatcgg
SEQ ID NO:149	52	-----gcagctactgt-----
SEQ ID NO:150	65	cgcttcttttcgcacaggaag-----ga
SEQ ID NO:151	22	ccctt--tc-----
SEQ ID NO:152	1	-----
SEQ ID NO:140	148	gcgaagttgaccgactggccgaacggatgcaggcagaggcaggagtgcc
SEQ ID NO:148	54	cc-----tggcaatcgccgagcggttccctgagcgagg-----
SEQ ID NO:149	63	-----ggaagcattc-----
SEQ ID NO:150	88	gctacgggtgatcg--ctggc-----gat-----
SEQ ID NO:151	29	-----
SEQ ID NO:152	1	-----gtgaacccaatgg---acaga--caaacagaaggacaag---
SEQ ID NO:140	198	ggcaaagcgcatcgatctcgaagtcagtgatgggagtgtatccggctcgcg
SEQ ID NO:148	86	-----gcg-----cgatgtcct-----
SEQ ID NO:149	73	-----gttaagggaagg-----
SEQ ID NO:150	109	-----atctcga-----
SEQ ID NO:151	29	-----
SEQ ID NO:152	35	-----aaccgcagc-----atcagg-----
SEQ ID NO:140	248	tacgtgccggtatcgaagcgattgtggcccgctcacggccagatcgacatt
SEQ ID NO:148	99	-----gatcgctcgcccggtgacgcc-----
SEQ ID NO:149	84	-----cgcttctgtagccttcgtg-----
SEQ ID NO:150	116	-----aagaaaatctcgactct
SEQ ID NO:151	29	-----cccgcca-----
SEQ ID NO:152	50	-----acagacagccgggcatt
SEQ ID NO:140	298	ctggtcaacaatgcaggaagtgccggtgccagcgctcgtctggccgagat
SEQ ID NO:148	118	-----gcc-----
SEQ ID NO:149	103	-----ggaagaaaccaagccaag-----
SEQ ID NO:150	133	cttgtgaaagaggcagaagg-----
SEQ ID NO:151	36	-----aaccaggaatgcc-----
SEQ ID NO:152	67	g-agtcaaaaatgaa-----tccgctgcc-----
SEQ ID NO:140	348	tccactcactgaagctgaattaggccctggcgccgaagagacgcttcag
SEQ ID NO:148	121	-----aagct-----cgaagccgcg-----g
SEQ ID NO:149	121	-----cttaag--gaagtag-----agagccgc-----tg
SEQ ID NO:150	153	-----
SEQ ID NO:151	51	-----
SEQ ID NO:152	90	-----

SEQ ID NO:140	398	ccagcatcgccaatttacttgggtatgggatggcatctgatgcgtattgcg
SEQ ID NO:148	138	ccagaagc-----tggcg
SEQ ID NO:149	144	ccagcagc-----
SEQ ID NO:150	153	-----actt-----
SEQ ID NO:151	51	-----cg
SEQ ID NO:152	90	-----gctgtcagaggacgaggattatc
SEQ ID NO:140	448	gcacctcatatgccggtaggaagtgcggtcatcaatgtctcgaccatctt
SEQ ID NO:148	151	gc-----tcttgcca---
SEQ ID NO:149	152	-----atggagccaacatc---
SEQ ID NO:150	157	-----ccgg--ggaag-----
SEQ ID NO:151	53	gcac-----
SEQ ID NO:152	113	g-----aggaa-----
SEQ ID NO:140	498	ttcacgggctgagtactacgggcggtatccgtatgtcaccctaaagctg
SEQ ID NO:148	162	-----ggc-----
SEQ ID NO:149	166	-----ctggctatcaaag-----cagatgtctcc---aaag---
SEQ ID NO:150	166	-----
SEQ ID NO:151	57	-----tac--cgatcggatgc-----agccg
SEQ ID NO:152	119	-----gcgg-----aaaactg
SEQ ID NO:140	548	ctcttaatgctctatctcaacttgctgcgctgagttaggtgcacgtggc
SEQ ID NO:148	165	-----cggcgc-----ggtggagacgtc
SEQ ID NO:149	194	-----acgagga
SEQ ID NO:150	166	-----
SEQ ID NO:151	76	c-----tgccgat-----cacgggg-
SEQ ID NO:152	130	aaaggaa-----aagttg-----
SEQ ID NO:140	598	atccgcgttaatacagatctttcccgccccgattgaaagtatcgcatccg
SEQ ID NO:148	183	gtccgc-----cgatcttgcc-----
SEQ ID NO:149	201	agc-----gaaaatcatcgta---
SEQ ID NO:150	166	-----gttgatccctacgtt-----ttgaacgtgaccg-----
SEQ ID NO:151	92	-----aaaac-----tcct
SEQ ID NO:152	143	-----cgatcattactgg-----
SEQ ID NO:140	648	tacagtgttcagcgtatggatcagctcaagggggcgcccgaaaggcgaca
SEQ ID NO:148	199	-----
SEQ ID NO:149	217	-----
SEQ ID NO:150	194	-acag-----ggatcagataaag-----gaag-----
SEQ ID NO:151	101	accagggttcc-----ggacgcctgaag-----
SEQ ID NO:152	156	-----aggcgaca
SEQ ID NO:140	698	cagcgcaccatttttgaacaccatgcgattgtgtcgtgccaacgaccag
SEQ ID NO:148	199	-----accag
SEQ ID NO:149	217	-----caacaa---
SEQ ID NO:150	215	-----ttgtggaaaa-----agtcgttcaaa---ag
SEQ ID NO:151	124	-----gacaag
SEQ ID NO:152	164	-----
SEQ ID NO:140	748	ggcgcgcttgaaacgtcggttcccctccgtcggatgtgtggcagacgccgc
SEQ ID NO:148	204	-----cct-----
SEQ ID NO:149	223	-----ac
SEQ ID NO:150	238	tacg-----gtcgaatc-----gatgt-----
SEQ ID NO:151	130	agagc-----catcatcaccggcgggga-----cagcggcatc
SEQ ID NO:152	164	-----

SEQ ID NO:140	798	tgtctttctggccagtgccgaatccgccgctctctccggtgagacgattg
SEQ ID NO:148	207	-----cggtgtcgcaaccgtcg-tcgagcaggtgaaa-----
SEQ ID NO:149	225	tgtc-----gacaagttc-----gggaagcttg
SEQ ID NO:150	255	-----tctggtga-----
SEQ ID NO:151	163	gg-----cagggccgtggcga-----tcgcc-----
SEQ ID NO:152	164	-----
SEQ ID NO:140	848	aggttacgcacggaatggagttgccggcctgcagtgagaccagcctgctg
SEQ ID NO:148	238	-----gagaccgggc-----
SEQ ID NO:149	248	atgt-----
SEQ ID NO:150	263	-----
SEQ ID NO:151	184	----tatgcgcgcgagggag-----c
SEQ ID NO:152	164	-----gcggaat-----agggagagc-----
SEQ ID NO:140	898	gcccgtactgatctgcgcacgattgatgccagtggccgcacgacgctcat
SEQ ID NO:148	248	-----ggccgctcgacattcct
SEQ ID NO:149	252	-----gcttggt-----aacaacgc-----
SEQ ID NO:150	263	-----acaacgc-----
SEQ ID NO:151	201	ggacgtccttatcagc-----tat
SEQ ID NO:152	180	-----
SEQ ID NO:140	948	ctgcgcggcgaccagattgaagaggtgatggcgctcaccggtatgttgc
SEQ ID NO:148	265	.at-----caacaatg-----ccggt-----
SEQ ID NO:149	267	-----
SEQ ID NO:150	270	-----
SEQ ID NO:151	220	ctgag-----cgagcatgacgacgcgatggccaccaaggct-----
SEQ ID NO:152	180	-----
SEQ ID NO:140	998	gtacctgtgggagtgaagtgatcatcggttccggttcgggtgcggcgctg
SEQ ID NO:148	280	-----gtcgcgcacctc
SEQ ID NO:149	267	-----tgggatt-----ctacggttcg-----
SEQ ID NO:150	270	-----gggaat-----
SEQ ID NO:151	256	----ctggtggag-gaag-----
SEQ ID NO:152	180	-----
SEQ ID NO:140	1048	gcccagttcgagcaggcagtcgaatgagagtcggcggtggccggcgcgaga
SEQ ID NO:148	292	gtgccgttcga-----gagcgtcagcg-----aggcgca--
SEQ ID NO:149	284	-----cgagtgt-----tctggagccga
SEQ ID NO:150	276	-----
SEQ ID NO:151	269	---caggtcgc-aaggccgt-----gcttgccgcggcgga
SEQ ID NO:152	180	-----agcag-----
SEQ ID NO:140	1098	ctttacgcctccattgccttgccactcgatccacgcgatccggcaacaa
SEQ ID NO:148	321	-----gttcagcactcc
SEQ ID NO:149	302	cttta-----ataca-----aactt
SEQ ID NO:150	276	-----aacia
SEQ ID NO:151	300	c-----atccagtcg-tccg---acca
SEQ ID NO:152	185	-----ctattgcctt-----
SEQ ID NO:140	1148	ttgacgctg--tcttcgattgggcccggcgagaataccggcggttcatg
SEQ ID NO:148	334	ttcgcgctc-----aatgtggcgg-----cggcg-----
SEQ ID NO:149	317	ttga-----
SEQ ID NO:150	281	gggatgc-----gcttcttg
SEQ ID NO:151	318	ttgccgcaggatcgtcgaaacggcgttcgggaactcggcggtcat----
SEQ ID NO:152	195	-----

```

SEQ ID NO:140 1196 cagcgggtgattctgcctgctaccagtcacgaaccggcaccgtgcgtgatt
SEQ ID NO:148 358 -----ttcttcct-----cacc-----
SEQ ID NO:149 321 -----tgaaact-----
SEQ ID NO:150 296 -----
SEQ ID NO:151 363 -----
SEQ ID NO:152 195 -----tgcta-----

SEQ ID NO:140 1246 gaggttgatgatgagcgggtgctgaattttctggccgatgaaatcaccgg
SEQ ID NO:148 370 -----caggggctgctgccgcatt-----
SEQ ID NO:149 328 -----atgaac-----acgaatttac--g
SEQ ID NO:150 296 -----tgag-----gatgaaa-----c
SEQ ID NO:151 363 -----
SEQ ID NO:152 200 -----aagagggggctga-----

SEQ ID NO:140 1296 gacaatttgattgccagtcgcctggccggttactggcagtcgcaacggc
SEQ ID NO:148 390 -----
SEQ ID NO:149 345 tccagttgtcctcatcactagcctg-----
SEQ ID NO:150 307 -----
SEQ ID NO:151 364 gaca-----
SEQ ID NO:152 213 -----

SEQ ID NO:140 1346 ttacccccggcgacgtgcgcgtgggcccgcgtgtcattttctctcgaac
SEQ ID NO:148 390 -----cggcgc-----c
SEQ ID NO:149 370 -----
SEQ ID NO:150 307 -----
SEQ ID NO:151 368 -----ttctcgtcaac
SEQ ID NO:152 213 -----tatctccattctat---ac

SEQ ID NO:140 1396 ggtgccgatcaaaatgggaatgtttacggacgcattcaaagtgcgctat
SEQ ID NO:148 397 ggtgc-----at
SEQ ID NO:149 370 -----gctat
SEQ ID NO:150 307 -----gaagaagactgggatg-----
SEQ ID NO:151 379 aatgc-----
SEQ ID NO:152 229 ttagacgagca-----ttcggacgca-----

SEQ ID NO:140 1446 cggtcagctcattcgtgtgtggcgtcacgaggtgaacttgactatcagc
SEQ ID NO:148 404 cgatca-----
SEQ ID NO:149 375 ccctcatttgatt-----gctacaaaaggag-----
SEQ ID NO:150 323 cggt-----aataaac
SEQ ID NO:151 384 -----
SEQ ID NO:152 250 -----gagg-----aac

SEQ ID NO:140 1496 gtgccagcgccgcccgtgatcatgtgctgccgcccgtatgggccaatcag
SEQ ID NO:148 410 -----
SEQ ID NO:149 402 -----
SEQ ID NO:150 334 gtg-----aatc--
SEQ ID NO:151 384 -----agcccatcag
SEQ ID NO:152 258 acgcaaacg-----gatc-----gaaaaggag

SEQ ID NO:140 1546 attgtgcgcttcgctaaccgcagccttgaagggttagaatttgctgtgc
SEQ ID NO:148 410 -----
SEQ ID NO:149 402 -----
SEQ ID NO:150 341 -----tgaagggt-----
SEQ ID NO:151 394 -----gcgaccttcaag-----
SEQ ID NO:152 280 aatgtccgctgc-----ctgcttatcc

```

```
SEQ ID NO:140 1596 ctggacagctcaattgctccatagtcacgccatatcaatgagattaccc
SEQ ID NO:148 410 -----
SEQ ID NO:149 402 -----catagttaacg---tatccagtata-----
SEQ ID NO:150 349 -----gttttcaacg-----
SEQ ID NO:151 406 -----
SEQ ID NO:152 302 cggga-----

SEQ ID NO:140 1646 tcaacatccctgccaacattagcgccaccaccggcgacgcagtgcatcg
SEQ ID NO:148 410 tcaacatctcttcttattt-----cgcccga-----
SEQ ID NO:149 424 -----ctgtctacaatag-----
SEQ ID NO:150 359 -----
SEQ ID NO:151 406 --aacatc---gaagacatcagcgac-----
SEQ ID NO:152 307 -----

SEQ ID NO:140 1696 gtcggatggcggaagcctgatcggttgcatattggggaaagttgcctt
SEQ ID NO:148 437 -----
SEQ ID NO:149 437 -----
SEQ ID NO:150 359 -----
SEQ ID NO:151 427 -----gagga-----
SEQ ID NO:152 307 ---gatg-----ttgggga-----

SEQ ID NO:140 1746 gattaccggtggcagcgccggtattggtgggcagatcgggcgccctcctgg
SEQ ID NO:148 437 -----
SEQ ID NO:149 437 -----
SEQ ID NO:150 359 -----
SEQ ID NO:151 432 -----gtggg-----
SEQ ID NO:152 318 -----

SEQ ID NO:140 1796 ctttgagtggcgcgcgctgatgctggcagcccgtgatcggcataagctc
SEQ ID NO:148 437 -----
SEQ ID NO:149 437 -----taa-----
SEQ ID NO:150 359 -----
SEQ ID NO:151 437 -----agctgacattccg-----c
SEQ ID NO:152 318 -----

SEQ ID NO:140 1846 gaacagatgcaggcgatgatccaatctgagctggctgaggtgggtatac
SEQ ID NO:148 437 -----agatgatcc-----
SEQ ID NO:149 440 -----gaatac
SEQ ID NO:150 359 -----tgactcagatgg-----
SEQ ID NO:151 451 gtcaacatgcagccatgttc-----tac
SEQ ID NO:152 318 -----cga-gaaccattgtgaacaagctg-----

SEQ ID NO:140 1896 cgatgtcgaagatcgcgctccacattgcaccgggctgcgatgtgagtagcg
SEQ ID NO:148 446 -----cg
SEQ ID NO:149 446 c-----
SEQ ID NO:150 371 -----
SEQ ID NO:151 475 c--tgaccaag-----gcagcgg-----
SEQ ID NO:152 341 -----tgca-----

SEQ ID NO:140 1946 aagcgcagcttgccgatcttgttgaacgtaccctgtcagcttttggcacc
SEQ ID NO:148 448 aagcg-----gccatc-----cagc
SEQ ID NO:149 447 -----
SEQ ID NO:150 371 -----
SEQ ID NO:151 491 -----tgccgcacatgaagaa-----gggcagc
SEQ ID NO:152 345 -----gcaaacagtggaacc-----attttggtaaa
```



```
SEQ ID NO:140 1996 gtcgattatctga-tcaacaacgccgggatcgccggtgtcgaagagatgg
SEQ ID NO:148 463 gtctactccctgt-ccaagggcgc-----
SEQ ID NO:149 447 -----
SEQ ID NO:150 371 -----
SEQ ID NO:151 514 g-----cga-tcatcaacaccg-----
SEQ ID NO:152 370 ctcgat-atcttagtgaacaacgccg-----

SEQ ID NO:140 2045 ttatcgatatgccagttgagggatggcgccataccctcttcgccaatctg
SEQ ID NO:148 486 -----gttga-----
SEQ ID NO:149 447 -----agggattatgtcatacagt-----
SEQ ID NO:150 371 -----
SEQ ID NO:151 530 -----cttcca-----tcaatgccgacgttcccaatccg
SEQ ID NO:152 395 -----ctg

SEQ ID NO:140 2095 atcagcaactactcgttgatgcgcaaactggcgccgttgatgaaaaaaca
SEQ ID NO:148 491 -----actcgttga-----
SEQ ID NO:149 466 -----
SEQ ID NO:150 371 -----tggtgccctacatgatcaaaca
SEQ ID NO:151 559 atc-----ctactcgccatgcg-----accacca
SEQ ID NO:152 398 aacagcatc-----ccca

SEQ ID NO:140 2145 gggtagcgggttacatcccttaacgtctcatcatactttggcgggtgaaaaag
SEQ ID NO:148 500 -----
SEQ ID NO:149 466 -----
SEQ ID NO:150 393 gaggaacgggtcgatcgtgaacgtctcctctgtcgttg-----aat
SEQ ID NO:151 584 agggcgcg-----atc-----cacaattt-----
SEQ ID NO:152 411 ggacag-----cattctcaatafttcaaca-----

SEQ ID NO:140 2195 atgcggccattccctaccccaaccgtgccgattacgccgtctcgaaggct
SEQ ID NO:148 500 -----ccagatcgt
SEQ ID NO:149 466 -----gtgtcaaaggct
SEQ ID NO:150 435 atacgggaat-----cctggtcagacgaattacgcggcgtcgaaggcg
SEQ ID NO:151 603 -----cagcgccg-----gtctcg-----
SEQ ID NO:152 436 -----

SEQ ID NO:140 2245 ggtcagcgggcaatggccgaagtctttgcgcgttctctggcccg---ga
SEQ ID NO:148 510 ggccttcgag-----ctcggcccgcgcg
SEQ ID NO:149 478 g-----
SEQ ID NO:150 478 ggagtcataggaatgacc-aagacgt-----
SEQ ID NO:151 617 ---cgcagatgctggccgaa-----cgcg---g-
SEQ ID NO:152 436 gaacagctggaa-----aaaacctttcgc-----

SEQ ID NO:140 2292 gatacagatcaatgccattgcgcgggtccgggtcgaaggatcgcttg
SEQ ID NO:148 534 catccgcgtcaacgccatcgcgcccggaacgggtcga-----
SEQ ID NO:149 479 -----
SEQ ID NO:150 503 -----gggcgaaggaaactcgct---
SEQ ID NO:151 639 gataagagtgaatgtcgtggccccgggcccgatc-----
SEQ ID NO:152 460 -acaaatattttttccat-----

SEQ ID NO:140 2342 gcggtaaccggtgaacgtcccgccctctttgcccgctcgggcgcggtgatt
SEQ ID NO:148 570 -----
SEQ ID NO:149 479 -----
SEQ ID NO:150 520 -----
SEQ ID NO:151 673 -----tggaacgcgctg---
SEQ ID NO:152 477 -----
```

SEQ ID NO:140 2392 ttggagaacaagcggctgaatgagcttcacgctgctcttatcgcggtgc
SEQ ID NO:148 570 -----
SEQ ID NO:149 479 -----
SEQ ID NO:150 520 -----ggaagaaacatcagggtaac-----gctgt
SEQ ID NO:151 685 -----atcccctccaccatgc-----
SEQ ID NO:152 477 -----gtttca-----

SEQ ID NO:140 2442 gcgcaccgatgagcgatctatgcacgaactggtgaactgctcttaccca
SEQ ID NO:148 570 -----
SEQ ID NO:149 479 -----ctatg---gatcacttcacaaaat-----
SEQ ID NO:150 546 g-gcacc-----cgga
SEQ ID NO:151 701 -----ccgagga-----
SEQ ID NO:152 483 -----tatg-acgaa-----

SEQ ID NO:140 2492 atgatgtggccgcactagagcagaatcccgcagcacctaccgcgttgctg
SEQ ID NO:148 570 -----cacc-----
SEQ ID NO:149 500 -----tggcagcgttgagctg-----gctccttctggcgtgcga
SEQ ID NO:150 556 ttcat-----agaaacccccatgac-----
SEQ ID NO:151 708 -----taccg-----
SEQ ID NO:152 492 -----gaaagctttgcct

SEQ ID NO:140 2542 gaactggcacgacgcttttcgcagcgaaggcgatccggcggcatcatcaag
SEQ ID NO:148 574 -----gcatgcggcg-----caag
SEQ ID NO:149 535 g-----
SEQ ID NO:150 576 -----cgaaaaacttcag-----aaaaag
SEQ ID NO:151 713 -----tcgccgatttcg-----
SEQ ID NO:152 505 cacctg-----caag

SEQ ID NO:140 2592 cagtgcgctgctgaaccggttcaattgccgctaaattgctggctcgtttgc
SEQ ID NO:148 589 -----accgt-----
SEQ ID NO:149 536 -----tgaac---tcagt-----
SEQ ID NO:150 596 c-----ccgtgaaacggcc-----
SEQ ID NO:151 725 -----gc
SEQ ID NO:152 515 aggggtg-----tgccatta-----

SEQ ID NO:140 2642 ataatggtggctatgtgttgctgccgacatctttgcaaacctgccaaac
SEQ ID NO:148 594 -----cgac-----aacctgcc-----
SEQ ID NO:149 546 -----caacctg-----
SEQ ID NO:150 610 -----ctttccaga-----
SEQ ID NO:151 727 aaacaggtgcctatg-----
SEQ ID NO:152 530 ttaat-----acgacat-----

SEQ ID NO:140 2692 ccgcccgatcccttcttcacccgagcccagattgatcgcgaggctcgcaa
SEQ ID NO:148 606 -----
SEQ ID NO:149 554 -----gaccagttct-----
SEQ ID NO:150 619 -----atacc-----gctgggaa
SEQ ID NO:151 742 -----aa
SEQ ID NO:152 542 -----cgattaccgctt-----

SEQ ID NO:140 2742 ggttcgtgacggcatcatggggatgctctacctgcaacggatgccgactg
SEQ ID NO:148 606 -----ggccga-----
SEQ ID NO:149 564 -----tac-----
SEQ ID NO:150 632 ggtttgggaagccagaagagg-----
SEQ ID NO:151 744 g-----
SEQ ID NO:152 554 -----atgaaggggat-----acgg-----

SEQ ID NO:140 2792 agtttgatgtcgcaatggccaccgtctattaccttgccgaccgcaatgtc
SEQ ID NO:148 612 -----ggcca-----agccgaactgaaggcc
SEQ ID NO:149 567 ----tgatatcgc-----
SEQ ID NO:150 653 -----tggcgca-----
SEQ ID NO:151 745 -----cgaccg-----
SEQ ID NO:152 569 -----cgттаattgattattccagcacaaag--

SEQ ID NO:140 2842 agtggtgagaca-ttccacccatcaggtggtttgcgttacgaacgcaccc
SEQ ID NO:148 634 tatg-----tcgaacgcagc-
SEQ ID NO:149 576 -----
SEQ ID NO:150 660 ---ggttatactcttcctcgcatcgacgagtcgagttacg-----
SEQ ID NO:151 751 -----
SEQ ID NO:152 595 ---ggtgcga-----ttgtttcctttacg-----

SEQ ID NO:140 2891 ctaccggtggcgaactcttcggcttgccctcaccggaacggctggcgag
SEQ ID NO:148 649 -----tatccgctgggcccgcacgg-ccgcccgcagcagc
SEQ ID NO:149 576 -----ag
SEQ ID NO:150 698 -----tcaccggacagg-
SEQ ID NO:151 751 -----ggccagccc-----gtggaa
SEQ ID NO:152 616 cgttccatggcgaagtc---gcttgc-----

SEQ ID NO:140 2941 ctggtcggaagcacggtctatctgataggtgaacatctgactgaacacct
SEQ ID NO:148 682 ctgcgcggcatggcggttatct-----
SEQ ID NO:149 578 ctggt-----tctggct-----
SEQ ID NO:150 710 -----tgatag-----
SEQ ID NO:151 766 ctcg-----cctcggcctatgtcat-----
SEQ ID NO:152 639 -----agataaa-----

SEQ ID NO:140 2991 taacctgcttgcccgctgcgtacctcgaacgttacggggcacgtcaggtag
SEQ ID NO:148 705 -----
SEQ ID NO:149 590 -----
SEQ ID NO:150 716 -----
SEQ ID NO:151 786 -----
SEQ ID NO:152 646 -----ggca-----

SEQ ID NO:140 3041 tgatgattggtgagacagaaaccggggcagagacaatgcgtcgcttgctc
SEQ ID NO:148 705 -----
SEQ ID NO:149 590 -----tttctc
SEQ ID NO:150 716 -----
SEQ ID NO:151 786 -----
SEQ ID NO:152 650 -----tcagagtgaatgcg-----

SEQ ID NO:140 3091 cacgatcacgtcgaggtggtcggtgatgactattgtggccggtgatca
SEQ ID NO:148 705 -----
SEQ ID NO:149 596 c-----tgatct
SEQ ID NO:150 716 -----
SEQ ID NO:151 786 -----gctgg-----
SEQ ID NO:152 664 -----gtggcgcccggt-----

SEQ ID NO:140 3141 gatcgaagccgctatcgaccaggctatcactcgctacggtcgcccagggc
SEQ ID NO:148 705 -----agccagcgacgaggc-----
SEQ ID NO:149 603 gcttgaag-----
SEQ ID NO:150 716 -----
SEQ ID NO:151 791 ---cggatccgatgtcga-----gctac-----
SEQ ID NO:152 676 -----ccgatttggacaccgct-----

```

SEQ ID NO:140 3191 cggtcgtctgtaccccccttcggccactgccgacgggtaccactggtcggg
SEQ ID NO:148 720 -----
SEQ ID NO:149 611 -----
SEQ ID NO:150 716 -----
SEQ ID NO:151 811 -----
SEQ ID NO:152 693 -----tattccgg-----cgacattccctgagg-----

SEQ ID NO:140 3241 cgtaaagacagtgactggagcacagtgttgagtgaggetgaatttgccga
SEQ ID NO:148 720 -----ggcctgga-----cga
SEQ ID NO:149 611 -----atacagg-----
SEQ ID NO:150 716 -----gaat-----
SEQ ID NO:151 811 -----gtgtcaggcgca-----
SEQ ID NO:152 716 -----aaaaagtga-aacagcac-----ggcttggtatccccca

SEQ ID NO:140 3291 gttgtgcgaacaccagctcaccaccatttcgggtagcgcgcaagattg
SEQ ID NO:148 731 gcggtgggatac-----tttg
SEQ ID NO:149 619 -----gctcatcacccgt-----
SEQ ID NO:150 720 -----
SEQ ID NO:151 823 -----acgattg
SEQ ID NO:152 748 ---atgggaagaccgggacagcc-----ggttgagc-----

SEQ ID NO:140 3341 ccctgagtgatgggtgc-cagtctcgcgctggctcactcccgaaactacggc
SEQ ID NO:148 746 .ccgtg---gatggt-----
SEQ ID NO:149 632 ---tggggaaagctgcgcagctc-----
SEQ ID NO:150 720 -----agatgg-----
SEQ ID NO:151 830 ccgtga-----
SEQ ID NO:152 776 -----atgcaggcgc-ctatgtctgctggcgctctgacgaa-----

SEQ ID NO:140 3390 tacctcaactaccgagcaatttgctctggctaacttcatacaaacgaccc
SEQ ID NO:148 757 -----
SEQ ID NO:149 652 -----gaggagattgct-----
SEQ ID NO:150 726 -----
SEQ ID NO:151 836 -----
SEQ ID NO:152 811 -----tcttcta-----

SEQ ID NO:140 3440 ttcacgcttttacggctacgattggtgtcgagagcgaaagaactgctcag
SEQ ID NO:148 757 -----ggcta-----
SEQ ID NO:149 664 -----gatatgatt-----
SEQ ID NO:150 726 -----
SEQ ID NO:151 836 -----
SEQ ID NO:152 819 -----tatga-----cag

SEQ ID NO:140 3490 cgcattctgatcaatcaagtcgatctgaccggcggtgcgcgtgccgaaga
SEQ ID NO:148 762 -----
SEQ ID NO:149 673 -----gtgtatctg-----gctagtataaagc
SEQ ID NO:150 726 -----gg
SEQ ID NO:151 836 -----
SEQ ID NO:152 827 ggca---gaccattcatgt-----gaatg

SEQ ID NO:140 3540 gccgcgtgatccgcacgagcgtcaacaagaactggaacgttttatcgagg
SEQ ID NO:148 762 -----
SEQ ID NO:149 696 taagagtgtt-----acggggctcctgttat-----
SEQ ID NO:150 728 gcctcgat-----
SEQ ID NO:151 836 -----
SEQ ID NO:152 848 gcggc-----cgtttat-----

```

```
SEQ ID NO:140 3590 cagtcttgctgggtcactgcaccactcccgctgaagccgatacccgttac
SEQ ID NO:148 762 -----
SEQ ID NO:149 721 ----atcatggacaatg---gactcgcgc-----
SEQ ID NO:150 738 -----ctga-----
SEQ ID NO:151 836 -----ccggcggcaagcc-----
SEQ ID NO:152 861 -----

SEQ ID NO:140 3640 gccgggcggattcatcgcggacgggcgattaccgtgtaa
SEQ ID NO:148 762 -----cacggccggatga-----
SEQ ID NO:149 743 -----tgca-----gtaa
SEQ ID NO:150 742 -----
SEQ ID NO:151 849 -----tttcctttga-
SEQ ID NO:152 861 -----ttcaac-----gtaa
```

Figure 54

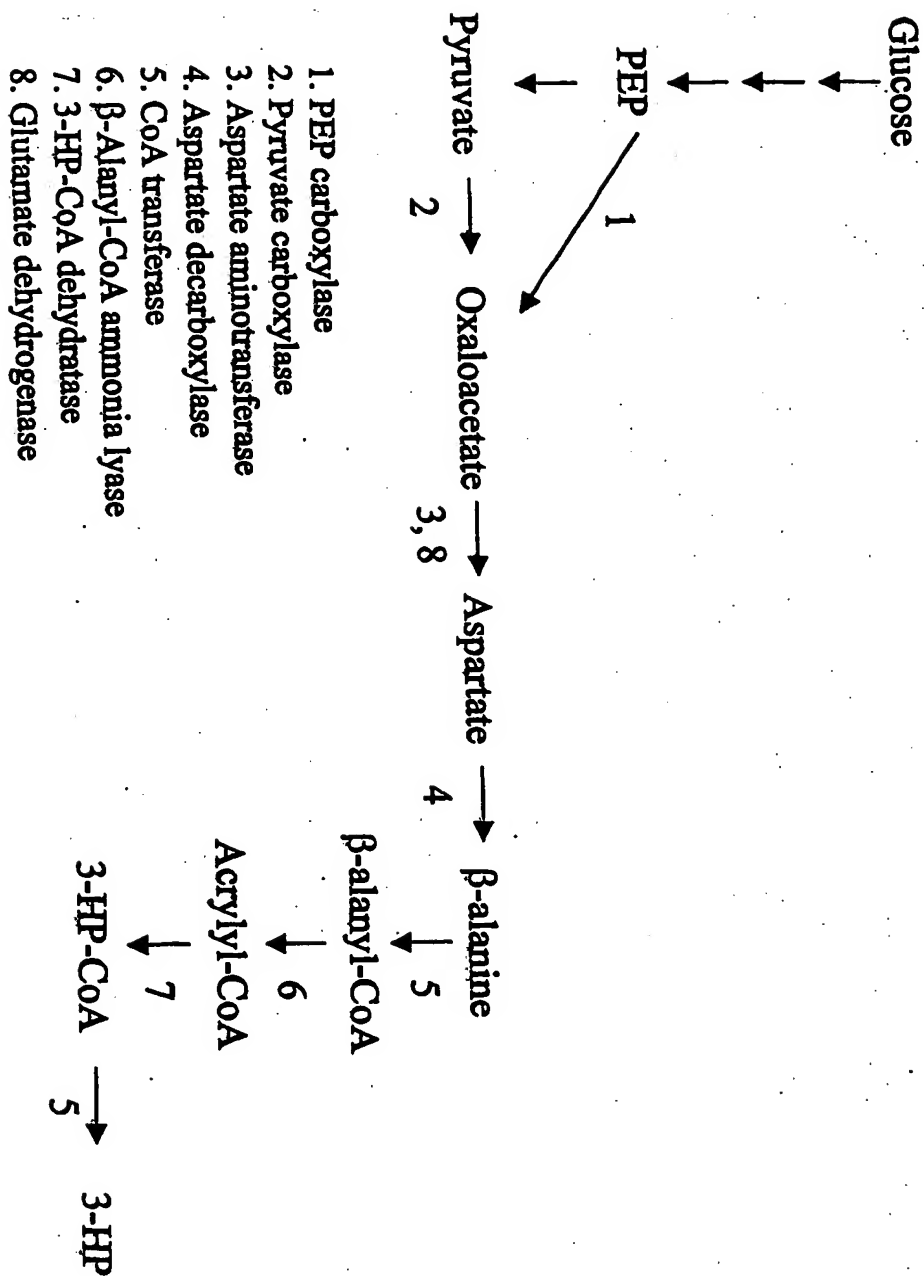


Figure 55

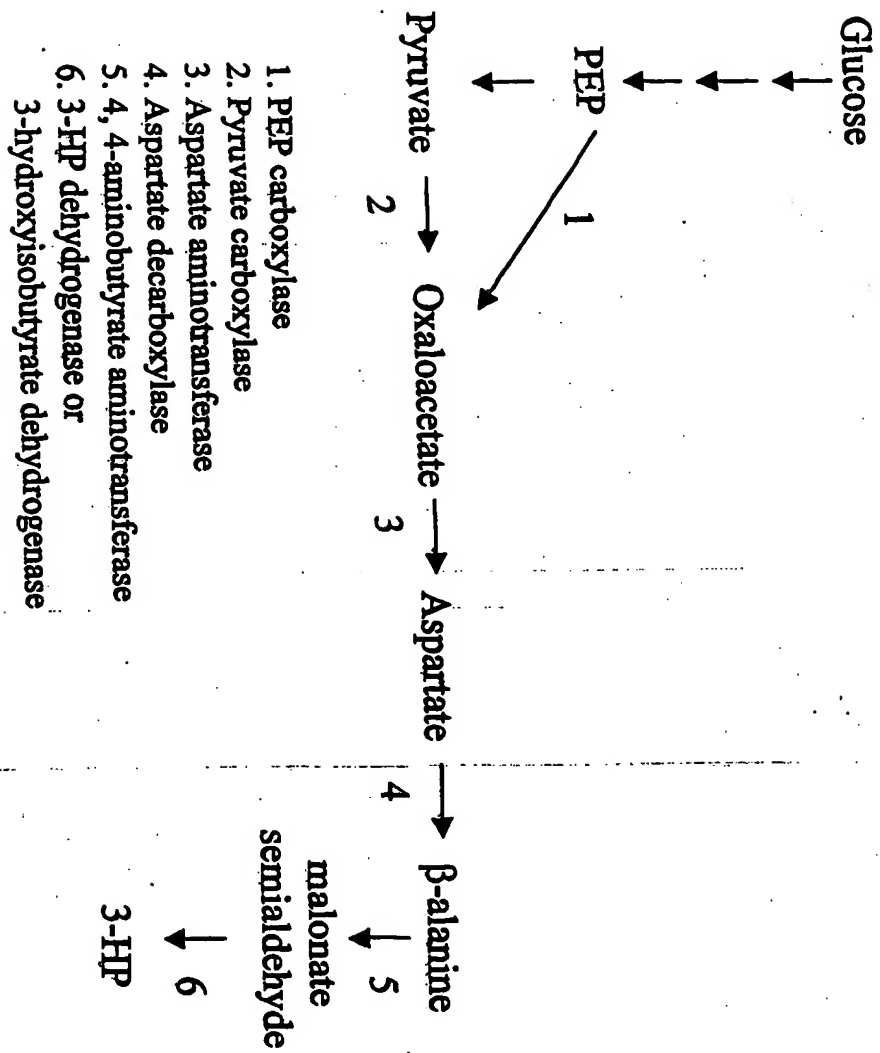


Figure 56

1 MVGKKVVHHL MMSAKDAHYT GNLVNGARIV NQWGDVGTEL
41 MYYVDGDISL FLGYKDIEFT APVYVGDFME YHGWIEKVGN
81 QSYTCKFEAW KVATMVDITN PQDTRATACE PPVLCGRATG
121 SLFIAKKDQR GPQESSFKER KHPGE (SEQ ID NO:160)

Figure 57

1 MVGKKVVHHL MMSAKDAHYT GNLVNGARIV NQWGDVGTEL
41 MVIYVGDISL FLGYKDIEFT APVYVGDFME YHGWIEKVG
81 QSYTCKFEAW KVAKMVDITN PQDTRATAACE PPVLCGTATG
121 SLFIKDNQR GPQESSFKDA KHPQ (SEQ ID NO:161)

Figure 58

```
1  ATGGTAGGTA AAAAGGTTGT ACATCATTTA ATGATGAGCG
41 CAAAAGATGC TCACTATACT GGAAACTTAG TAAACGGCGC
81 TAGAATTGTG AATCAGTGGG GCGACGTTGG TACAGAATTA
121 ATGGTTTATG TTGATGGTGA CATAAGCTTA TTCTTGGGCT
161 ACAAAGATAT CGAATTCACA GCTCCTGTAT ATGTTGGTGA
201 CTTTATGGAA TACCAGGGCT GGATTGAAAA AGTTGGTAAC
241 CAGTCCTATA CATGTAAATT TGAAGCATGG AAAGTTGCAA
281 CAATGGTTGA TATCACAAT CCTCAGGATA CACGGGCAAC
321 AGCTTGTGAG CCTCCGGTAT TGTGCGGAAG AGCAACGGGT
361 AGTTTGTTC TCGCAAAAAA AGATCAGAGA GGCCCTCAGG
401 AATCCTCTTT TAAAGAGAGA AAGCACCCCG GTGAATGA
(SEQ ID NO:162)
```

Figure 59

```
1  ATGGTAGGTA AAAAGGTTGT ACATCATTTA ATGATGAGCG
41  CAAAAGATGC TCACTATACT GGAAACTTAG TAAACGGCGC
81  TAGAATTGTG AATCAGTGGG GCGACGTAGG TACAGAATTA
121 ATGGTTTATG TTGATGGTGA CATCAGCTTA TTCTTGGGCT
161 ACAAAGATAT CGAATTCACA GCTCCTGTAT ATGTTGGTGA
201 TTTTATGGAA TACCAGGGCT GGATTGAAAA AGTTGGCAAC
241 CAGTCCTATA CATGTAAATT TGAAGCATGG AAAGTAGCAA
281 AGATGGTTGA TATCACAAAT CCACAGGATA CACGTGCAAC
321 AGCTTGTGAA CCTCCGGTAC TTTGTGGTAC TGCAACAGGC
361 AGCCTTTTCA TCGCAAAGGA TAATCAGAGA GGTCCCTCAGG
401 AATCTTCCTT CAAGGATGCA AAGCACCTC AATAA
(SEQ ID NO:163)
```



CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 May 2002 (30.05.2002)

PCT

(10) International Publication Number
WO 02/042418 A2

(51) International Patent Classification⁷: C12N

Brooklyn Park, MN 55429 (US). SELMER, Thorsten [DE/DE]; Cappeler Strasse 12, 35039 Marburg (DE). BUCKEL, Wolfgang [DE/DE]; Am Koeppel 8, 35043 Marburg (DE).

(21) International Application Number: PCT/US01/43607

(22) International Filing Date:
20 November 2001 (20.11.2001)

(74) Agent: DEGRANDIS, Paula; Cargill, Incorporated, P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/252,123 20 November 2000 (20.11.2000) US
60/285,478 20 April 2001 (20.04.2001) US
60/306,727 20 July 2001 (20.07.2001) US
60/317,845 7 September 2001 (07.09.2001) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US 60/252,123 (CON)
Filed on 20 November 2000 (20.11.2000)
US 60/285,478 (CON)
Filed on 20 April 2001 (20.04.2001)
US 60/306,727 (CON)
Filed on 20 July 2001 (20.07.2001)
US 60/317,845 (CON)
Filed on 7 September 2001 (07.09.2001)

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

(71) Applicant (*for all designated States except US*):
CARGILL, INCORPORATED [US/US]; 15407
McGinty Road West, Wayzata, MN 55391-2399 (US).

(48) Date of publication of this corrected version:
21 November 2002

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): GOKARN, Ravi, R. [IN/US]; 3205 Harbor, Lane #4311, Plymouth, MN 55447 (US). SELIFONOVA, Olga, V. [RU/US]; 1405 Olive Lane N. #318, Plymouth, MN 55447 (US). JESSEN, Holly [US/US]; 6618 Brenden Court, Chanhassen, MN 55317 (US). GORT, Steven, J. [US/US]; 3207 Quarles Road,

(15) Information about Correction:
see PCT-Gazette No. 47/2002 of 21 November 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/042418 A2

(54) Title: 3-HYDROXYPROPIONIC ACID AND OTHER ORGANIC COMPOUNDS

(57) Abstract: The invention provides methods and materials related to producing 3-HP as well as other organic compounds. Specifically, the invention provides isolated nucleic acids, polypeptides, host cells, and methods and materials for producing 3-HP and other organic compounds.

5

3-HYDROXYPROPIONIC ACID AND OTHER ORGANIC COMPOUNDS

FIELD OF THE INVENTION

The invention relates to enzymes and methods that can be used to produce organic
10 acids and related products.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from the following U.S. Provisional Patent
Applications, which are herein incorporated by reference: U.S. Provisional Patent
15 Application Serial Number 60/252,123, filed November 20, 2000; U.S. Provisional Patent
Application Serial Number 60/285,478, filed April 20, 2001; U.S. Provisional Patent
Application Serial Number 60/306,727, filed July 20, 2001; and U.S. Provisional Patent
Application Serial Number 60/317,845, filed September 7, 2001.

20

BACKGROUND

Organic chemicals such as organic acids, esters, and polyols can be used to
synthesize plastic materials and other products. To meet the increasing demand for
organic chemicals, more efficient and cost effective production methods are being
developed which utilize raw materials based on carbohydrates rather than hydrocarbons.
25 For example, certain bacteria have been used to produce large quantities of lactic acid
used in the production of polylactic acid.

3-hydroxypropionic acid (3-HP) is an organic acid. Although several chemical
synthesis routes have been described to produce 3-HP, only one biocatalytic route has
been heretofore previously disclosed (WO 01/16346 to Suthers, et al.). 3-HP has utility
30 for specialty synthesis and can be converted to commercially important intermediates by
known art in the chemical industry, e.g., acrylic acid by dehydration, malonic acid by

oxidation, esters by esterification reactions with alcohols, and reduction to 1,3 propanediol.

SUMMARY

5 The invention relates to methods and materials involved in producing 3-hydroxypropionic acid and other organic compounds (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, esters of 3-HP, and malonic acid and its esters). Specifically, the invention provides nucleic acid molecules, polypeptides, host cells, and methods that can be used to produce 3-HP and other organic
10 compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, esters of 3-HP, and malonic acid and its esters. 3-HP has potential to be both biologically and commercially important. For example, the nutritional industry can use 3-HP as a food, feed additive or preservative, while the derivatives mentioned above can be produced from 3-HP. The nucleic acid molecules described herein can be
15 used to engineer host cells with the ability to produce 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP. The polypeptides described herein can be used in cell-free systems to make 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and
20 esters of 3-HP. The host cells described herein can be used in culture systems to produce large quantities of 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP.

One aspect of the invention provides cells that have lactyl-CoA dehydratase
25 activity and 3-hydroxypropionyl-CoA dehydratase activity, and methods of making products such as those described herein by culturing at least one of the cells that have lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity. In some embodiments, the cell can also contain an exogenous nucleic acid molecule that encodes one or more of the following polypeptides: a polypeptide having E1 activator
30 activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-

- CoA dehydratase activity; and a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. Additionally, the cell can have CoA transferase activity, CoA synthetase activity, poly hydroxyacid synthase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, and/or lipase activity.
- 5 Moreover, the cell can contain at least one exogenous nucleic acid molecule that expresses one or more polypeptides that have CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, CoA synthetase activity, poly hydroxyacid synthase activity, and/or lipase activity.

- In another embodiment of the invention, the cell that has lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity produces a product, for example, 3-HP, polymerized 3-HP, and/or an ester of 3-HP, such as methyl hydroxypropionate, ethyl hydroxypropionate, propyl hydroxypropionate, and/or butyl hydroxypropionate. Accordingly, the invention also provides methods of producing one or more of these products. These methods involve culturing the cell that has lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity under conditions
- 10 activity and 3-hydroxypropionyl-CoA dehydratase activity produces a product, for example, 3-HP, polymerized 3-HP, and/or an ester of 3-HP, such as methyl hydroxypropionate, ethyl hydroxypropionate, propyl hydroxypropionate, and/or butyl hydroxypropionate. Accordingly, the invention also provides methods of producing one or more of these products. These methods involve culturing the cell that has lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity under conditions
- 15 that allow the product to be produced. These cells also can have CoA synthetase activity and/or poly hydroxyacid synthase activity.

- Another aspect of the invention provides cells that have CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity. In some embodiments, these cells also can contain an exogenous nucleic acid molecule that encodes one or more of the following polypeptides: a polypeptide having E1 activator activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; a polypeptide having CoA synthetase activity; and a
- 20 embodiments, these cells also can contain an exogenous nucleic acid molecule that encodes one or more of the following polypeptides: a polypeptide having E1 activator activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; a polypeptide having CoA synthetase activity; and a
- 25 polypeptide having poly hydroxyacid synthase activity.

In another embodiment of the invention, the cell that has CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity can produce a product, for example, polymerized acrylate.

- Another aspect of the invention provides a cell comprising CoA transferase activity, lactyl-CoA dehydratase activity, and lipase activity. In some embodiments, the cell also can contain an exogenous nucleic acid molecule that encodes one or more of the
- 30 activity, lactyl-CoA dehydratase activity, and lipase activity. In some embodiments, the cell also can contain an exogenous nucleic acid molecule that encodes one or more of the

following polypeptides: a polypeptide having CoA transferase activity; a polypeptide having E1 activator activity; an E2 α polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; an E2 β polypeptide that is a subunit of an enzyme having lactyl-CoA dehydratase activity; and a polypeptide having lipase activity. This cell can be used, among other things, to produce products such as esters of acrylate (e.g., methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate).

In some embodiments, 1,3 propanediol can be created from either 3-HP-CoA or 3-HP via the use of polypeptides having enzymatic activity. These polypeptides can be used either *in vitro* or *in vivo*. When converting 3-HP-CoA to 1,3 propanediol, polypeptides having oxidoreductase activity or reductase activity (e.g., enzymes from the 1.1.1.- class of enzymes) can be used. Alternatively, when creating 1,3 propanediol from 3-HP, a combination of (1) a polypeptide having aldehyde dehydrogenase activity (e.g., an enzyme from the 1.1.1.34 class) and (2) a polypeptide having alcohol dehydrogenase activity (e.g., an enzyme from the 1.1.1.32 class) can be used.

In some embodiments of the invention, products are produced *in vitro* (outside of a cell). In other embodiments of the invention, products are produced using a combination of *in vitro* and *in vivo* (within a cell) methods. In yet other embodiments of the invention, products are produced *in vivo*. For methods involving *in vivo* steps, the cells can be isolated cultured cells or whole organisms such as transgenic plants, non-human mammals, or single-celled organisms such as yeast and bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells). Hereinafter such cells are referred to as production cells. Products produced by these production cells can be organic products such as 3-HP and/or the nucleic acid molecules and polypeptides described herein.

Another aspect of the invention provides polypeptides having an amino acid sequence that (1) is set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161, (2) is at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161, (3) has at least 65 percent sequence identity with at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161, (4) is a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 having conservative amino acid substitutions,

or (5) has at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. Accordingly, the invention also provides nucleic acid sequences that encode any of the polypeptides described herein as well as specific binding agents that bind to any of the polypeptides described herein. Likewise, 5 the invention provides transformed cells that contain any of the nucleic acid sequences that encode any of the polypeptides described herein. These cells can be used to produce nucleic acid molecules, polypeptides, and organic compounds. The polypeptides can be used to catalyze the formation of organic compounds or can be used as antigens to create specific binding agents.

10 In yet another embodiment, the invention provides isolated nucleic acid molecules that contain at least one of the following nucleic acid sequences: (1) a nucleic acid sequence as set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; (2) a nucleic acid sequence having at least 10 consecutive nucleotides from a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, 15 or 163; (3) a nucleic acid sequences that hybridize under hybridization conditions (e.g., moderately or highly stringent hybridization conditions) to a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; (4) a nucleic acid sequence having 65 percent sequence identity with at least 10 consecutive nucleotides from a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 20 142, 162, or 163; and (5) a nucleic acid sequence having at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. Accordingly, the invention also provides a production cell that contains at least one exogenous nucleic acid having any the nucleic acid sequences provided above. The production cell can be used to express polypeptides that have an 25 enzymatic activity such as CoA transferase activity, lactyl-CoA dehydratase activity, CoA synthase activity, dehydratase activity, dehydrogenase activity, malonyl CoA reductase activity, β -alanine ammonia lyase activity, and/or 3-hydroxypropionyl-CoA dehydratase activity. Accordingly, the invention also provides methods of producing polypeptides encoded by the nucleic acid sequences described above.

30 The invention also provides several methods such as methods for making 3-HP from lactate, phosphoenolpyruvate (PEP), or pyruvate. In some embodiments, methods

for making 3-HP from lactate, PEP, or pyruvate involve culturing a cell containing at least one exogenous nucleic acid under conditions that allow the cell to produce 3-HP. These methods can be practiced using the various types of production cells described herein. In some embodiments, the production cells can have one or more of the following activities: CoA transferase activity, 3-hydroxypropionyl-CoA hydrolase activity, 3-hydroxyisobutryl-CoA hydrolase activity, dehydratase activity, and/or malonyl CoA reductase activity.

In other embodiments, the methods involve making 3-HP wherein lactate is contacted with a first polypeptide having CoA transferase activity or CoA synthetase activity such that lactyl-CoA is formed, then contacting lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, then contacting acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA, and then contacting 3-hydroxypropionic acid-CoA with the first polypeptide to form 3-HP or with a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form 3-HP.

Another aspect of the invention provides methods for making polymerized 3-HP. These methods involve making 3-hydroxypropionic acid-CoA as described above, and then contacting the 3-hydroxypropionic acid-CoA with a polypeptide having polyhydroxyacid synthase activity to form polymerized 3-HP.

In yet another embodiment of the invention, methods for making an ester of 3-HP are provided. These methods involve making 3-HP as described above, and then additionally contacting 3-HP with a fifth polypeptide having lipase activity to form an ester.

The invention also provides methods for making polymerized acrylate. These methods involve culturing a cell that has both CoA synthetase activity, lactyl-CoA dehydratase activity, and polyhydroxyacid synthase activity such that polymerized acrylate is made. Accordingly, the invention also provides methods of making polymerized acrylate wherein lactate is contacted with a first polypeptide having CoA synthetase activity to form lactyl-CoA, then contacting lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, and then

contacting acrylyl-CoA with a third polypeptide having poly hydroxyacid synthase activity to form polymerized acrylate.

The invention also provides methods of making an ester of acrylate. These methods involve culturing a cell that has CoA transferase activity, lipase activity, and
5 lactyl-CoA dehydratase activity under conditions that allow the cell to produce an ester.

In another embodiment, the invention provides methods for making an ester of acrylate, wherein acrylyl-CoA is formed as described above, and then acrylyl-CoA is contacted with a polypeptide having CoA transferase activity to form acrylate, and acrylate is contacted with a polypeptide having lipase activity to form the ester.

10 The invention also provides methods for making 3-HP. These methods involve culturing a cell containing at least one exogenous nucleic acid that encodes at least one polypeptide such that 3-HP is produced from acetyl-CoA or malonyl-CoA.

Alternative embodiments provide methods of making 3-HP, wherein acetyl-CoA is contacted with a first polypeptide having acetyl-CoA carboxylase activity to form
15 malonyl-CoA, and malonyl-CoA is contacted with a second polypeptide having malonyl-CoA reductase activity to form 3-HP.

In other embodiments, malonyl-CoA can be contacted with a polypeptide having malonyl-CoA reductase activity so that 3-HP can be made.

In another embodiment, the invention provides a method for making 3-HP that
20 uses a β -alanine intermediate. This method can be performed by contacting β -alanine CoA with a first polypeptide having β -alanyl-CoA ammonia lyase activity (such as a polypeptide having the amino acid sequence set forth in SEQ ID NO: 160 or 161) to form acrylyl-CoA, contacting acrylyl-CoA with a second polypeptide having 3-HP-CoA dehydratase activity to form 3-HP-CoA, and contacting 3-HP-CoA with a third
25 polypeptide having glutamate dehydrogenase activity to make 3-HP.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable
30 methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In

case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

5

DESCRIPTION OF DRAWINGS

Figure 1 is a diagram of a pathway for making 3-HP.

Figure 2 is a diagram of a pathway for making polymerized 3-HP.

Figure 3 is a diagram of a pathway for making esters of 3-HP.

10 Figure 4 is a diagram of a pathway for making polymerized acrylic acid.

Figure 5 is a diagram of a pathway for making esters of acrylate.

Figure 6 is a listing of a nucleic acid sequence that encodes a polypeptide having CoA transferase activity (SEQ ID NO:1).

15 Figure 7 is a listing of an amino acid sequence of a polypeptide having CoA transferase activity (SEQ ID NO:2).

Figure 8 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:1, 3, 4, and 5.

Figure 9 is an alignment of the amino acid sequences set forth in SEQ ID NOs:2, 6, 7, and 8.

20 Figure 10 is a listing of a nucleic acid sequence that encodes a polypeptide having E1 activator activity (SEQ ID NO:9).

Figure 11 is a listing of an amino acid sequence of a polypeptide having E1 activator activity (SEQ ID NO:10).

25 Figure 12 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:9, 11, 12, and 13.

Figure 13 is an alignment of the amino acid sequences set forth in SEQ ID NOs:10, 14, 15, and 16.

Figure 14 is a listing of a nucleic acid sequence that encodes an E2 α subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:17).

30 Figure 15 is a listing of an amino acid sequence of an E2 α subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:18).

Figure 16 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:17, 19, 20, and 21.

Figure 17 is an alignment of the amino acid sequences set forth in SEQ ID NOs:18, 22, 23, and 24.

5 Figure 18 is a listing of a nucleic acid sequence that encodes an E2 β subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:25). The "G" at position 443 can be an "A"; and the "A" at position 571 can be a "G".

Figure 19 is a listing of an amino acid sequence of an E2 β subunit of an enzyme having lactyl-CoA dehydratase activity (SEQ ID NO:26).

10 Figure 20 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:25, 27, 28, and 29.

Figure 21 is an alignment of the amino acid sequences set forth in SEQ ID NOs:26, 30, 31, and 32.

15 Figure 22 is a listing of a nucleic acid sequence of genomic DNA from *Megasphaera elsdenii* (SEQ ID NO:33).

Figure 23 is a listing of a nucleic acid sequence that encodes a polypeptide from *Megasphaera elsdenii* (SEQ ID NO:34).

Figure 24 is a listing of an amino acid sequence of a polypeptide from *Megasphaera elsdenii* (SEQ ID NO:35).

20 Figure 25 is a listing of a nucleic acid sequence that encodes a polypeptide having enzymatic activity (SEQ ID NO:36).

Figure 26 is a listing of an amino acid sequence of a polypeptide having enzymatic activity (SEQ ID NO:37).

25 Figure 27 is a listing of a nucleic acid sequence that contains non-coding as well as coding sequence of a polypeptide having CoA synthase, dehydratase, and dehydrogenase activity (SEQ ID NO:38). The start site for the coding sequence is at position 480, a ribosome binding site is at position 466-473, and the stop codon is at position 5946.

30 Figure 28 is a listing of an amino acid sequence from a polypeptide having CoA synthase, dehydratase, and dehydrogenase activity (SEQ ID NO:39).

Figure 29 is a listing of a nucleic acid sequence that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (SEQ ID NO:40).

Figure 30 is a listing of an amino acid sequence of a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (SEQ ID NO:41).

5 Figure 31 is a listing of a nucleic acid sequence that contains non-coding as well as coding sequence of a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (SEQ ID NO:42).

Figure 32 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs:40, 43, 44, and 45.

10 Figure 33 is an alignment of the amino acid sequences set forth in SEQ ID NOs:41, 46, 47, and 48.

Figure 34 is a diagram of the construction of a synthetic operon (pTDH) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

15 Figure 35A and B is a diagram of the construction of a synthetic operon (pHTD) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

20 Figure 36A and B is a diagram of the construction of a synthetic operon (pEIITHrEI) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

Figure 37A and B is a diagram of the construction of a synthetic operon (pEIITHEI) that encodes for polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E1, E2 α , and E2 β), and 3-hydroxypropionyl-CoA dehydratase activity (3-HP-CoA dehydratase).

25 Figure 38A and B is a diagram of the construction of two plasmids, pEIITH and pPROEI. The pEIITH plasmid encodes polypeptides having CoA transferase activity, lactyl-CoA dehydratase activity (E2 α and E2 β), and 3-hydroxypropionyl-CoA

30

dehydratase activity (3-HP-CoA dehydratase), and the pPROEI plasmid encodes a polypeptide having E1 activator activity.

Figure 39 is a listing of a nucleic acid sequence that encodes a polypeptide having CoA synthase, dehydratase, and dehydrogenase activity (SEQ ID NO:129).

5 Figure 40 is an alignment of the amino acid sequences set forth in SEQ ID NOs:39, 130, and 131. The uppercase amino acid residues represent positions where that amino acid residue is present in two or more sequences.

Figure 41 is an alignment of the amino acid sequences set forth in SEQ ID NOs:39, 132, and 133. The uppercase amino acid residues represent positions where that amino acid residue is present in two or more sequences.

Figure 42 is an alignment of the amino acid sequences set forth in SEQ ID NOs: 39, 134, and 135. The uppercase amino acid residues represent positions where that amino acid residue is present in two or more sequences.

Figure 43 is a diagram of several pathways for making organic compounds using the multifunctional OS17 enzyme.

Figure 44 is a diagram of a pathway for making 3-HP via acetyl-CoA and malonyl-CoA.

Figure 45 is a diagram of pMSD8, pET30a/acc1, pFN476, and PET286 constructs.

Figure 46 contains a total ion chromatogram and five mass spectrums of Coenzyme A thioesters. Panel A is total ion chromatogram illustrating the separation of Coenzyme A and four CoA-organic thioesters: 1=Coenzyme A, 2=lactyl-CoA, 3=acetyl-CoA, 4=acrylyl-CoA, 5=propionyl-CoA. Panel B is a mass spectrum of Coenzyme A. Panel C is a mass spectrum of lactyl-CoA. Panel D is a mass spectrum of acetyl-CoA. Panel E is a mass spectrum of acrylyl-CoA. Panel F is a mass spectrum of propionyl-CoA.

Figure 47 contains ion chromatograms and mass spectrums. Panel A is a total ion chromatogram of a mixture of lactyl-CoA and 3-HP-CoA. The Panel A insert is the mass spectrum recorded under peak 1. Panel B is a total ion chromatogram of lactyl-CoA. The Panel B insert is the mass spectrum recorded under peak 2. In each panel, peak 1 is 3-HP-CoA, and peak 2 is lactyl-CoA. The peak labeled with an asterisk was confirmed not to be a CoA ester.

Figure 48 contains ion chromatograms and mass spectrums. Panel A is a total ion chromatogram of CoA esters derived from a broth produced by *E. coli* transfected with pEIITHrEI. The Panel A insert is the mass spectrum recorded under peak 1. Panel B is a total ion chromatogram of CoA esters derived from a broth produced by control *E. coli* not transfected with pEIITHrEI. The Panel B insert is the mass spectrum recorded under peak 2. In each panel, peak 1 is 3-HP-CoA, and peak 2 is lactyl-CoA. The peaks labeled with an asterisk were confirmed not to be a CoA ester.

Figure 49 is a listing of a nucleic acid sequence that encodes a polypeptide having malonyl-CoA reductase activity (SEQ ID NO: 140).

Figure 50 is a listing of an amino acid sequence of a polypeptide having malonyl-CoA reductase activity (SEQ ID NO:141).

Figure 51 is a listing of a nucleic acid sequence that encodes a portion of a polypeptide having malonyl-CoA reductase activity (SEQ ID NO:142).

Figure 52 is an alignment of the amino acid sequences set forth in SEQ ID NOs: 141, 143, 144, 145, 146, and 147.

Figure 53 is an alignment of the nucleic acid sequences set forth in SEQ ID NOs: 140, 148, 149, 150, 151, and 152.

Figure 54 is a diagram of a pathway for making 3-HP via a β -alanine intermediate.

Figure 55 is a diagram of a pathway for making 3-HP via a β -alanine intermediate.

Figure 56 is a listing of an amino acid sequence of a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:160).

Figure 57 is a listing of an amino acid sequence of a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:161).

Figure 58 is a listing of a nucleic acid sequence that encodes a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:162).

Figure 59 is a listing of a nucleic acid sequence that can encode a polypeptide having β -alanyl-CoA ammonia lyase activity (SEQ ID NO:163).

DETAILED DESCRIPTION

I. Terms

Nucleic acid: The term "nucleic acid" as used herein encompasses both RNA and DNA including, without limitation, cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear.

Isolated: The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously-replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or

eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

It will be apparent to those of skill in the art that a nucleic acid existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be
5 considered an isolated nucleic acid.

Exogenous: The term "exogenous" as used herein with reference to nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Thus, non-naturally-occurring nucleic acid is considered to be
10 exogenous to a cell once introduced into the cell. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of person X is an exogenous nucleic acid with respect to a cell of person Y once that chromosome is introduced into Y's cell.

Hybridization: The term "hybridization" as used herein refers to a method of
15 testing for complementarity in the nucleotide sequence of two nucleic acid molecules, based on the ability of complementary single-stranded DNA and/or RNA to form a duplex molecule. Nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, any nucleic acid having some homology to a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129,
20 140, 142, 162, or 163 can be used as a probe to identify a similar nucleic acid by hybridization under conditions of moderate to high stringency. Once identified, the nucleic acid then can be purified, sequenced, and analyzed to determine whether it is within the scope of the invention as described herein.

Hybridization can be done by Southern or Northern analysis to identify a DNA or
25 RNA sequence, respectively, that hybridizes to a probe. The probe can be labeled with a biotin, digoxigenin, an enzyme, or a radioisotope such as ³²P. The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art such as those described in sections
30 7.39-7.52 of Sambrook et al., (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY. Typically, a probe is at least about 20 nucleotides in

length. For example, a probe corresponding to a 20 nucleotide sequence set forth in SEQ ID NO: 1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, or 142 can be used to identify an identical or similar nucleic acid. In addition, probes longer or shorter than 20 nucleotides can be used.

5 The invention also provides isolated nucleic acid sequences that are at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000 bases in length) and hybridize, under hybridization conditions, to the sense or antisense strand of a nucleic acid having the sequence set forth in SEQ ID NO: 1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142,
10 162, or 163. The hybridization conditions can be moderately or highly stringent hybridization conditions.

 For the purpose of this invention, moderately stringent hybridization conditions mean the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO₄ (pH 7.4), 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated
15 salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about 5x10⁷ cpm/µg), while the washes are performed at about 50°C with a wash solution containing 2X SSC and 0.1% sodium dodecyl sulfate.

 Highly stringent hybridization conditions mean the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO₄ (pH 7.4), 5X SSC, 5X
20 Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about 5x10⁷ cpm/µg), while the washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% sodium dodecyl sulfate.

Purified: The term "purified" as used herein does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified polypeptide or
25 nucleic acid preparation can be one in which the subject polypeptide or nucleic acid, respectively, is at a higher concentration than the polypeptide or nucleic acid would be in its natural environment within an organism. For example, a polypeptide preparation can be considered purified if the polypeptide content in the preparation represents at least
30 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 98%, or 99% of the total protein content of the preparation.

Transformed: A "transformed" cell is a cell into which a nucleic acid molecule has been introduced by, for example, molecular biology techniques. As used herein, the term "transformation" encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell including, without limitation, transfection with a viral vector, conjugation, transformation with a plasmid vector, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Recombinant: A "recombinant" nucleic acid is one having (1) a sequence that is not naturally occurring in the organism in which it is expressed or (2) a sequence made by an artificial combination of two otherwise-separated, shorter sequences. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. "Recombinant" is also used to describe nucleic acid molecules that have been artificially manipulated, but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated.

Specific binding agent: A "specific binding agent" is an agent that is capable of specifically binding to any of the polypeptide described herein, and can include polyclonal antibodies, monoclonal antibodies (including humanized monoclonal antibodies), and fragments of monoclonal antibodies such as Fab, F(ab')₂, and Fv fragments as well as any other agent capable of specifically binding to an epitope of such polypeptides.

Antibodies to the polypeptides provided herein (or fragments thereof) can be used to purify or identify such polypeptides. The amino acid and nucleic acid sequences provided herein allow for the production of specific antibody-based binding agents that recognize the polypeptides described herein.

Monoclonal or polyclonal antibodies can be produced to the polypeptides, portions of the polypeptides, or variants thereof. Optimally, antibodies raised against one or more epitopes on a polypeptide antigen will specifically detect that polypeptide. That is, antibodies raised against one particular polypeptide would recognize and bind that particular polypeptide, and would not substantially recognize or bind to other polypeptides. The determination that an antibody specifically binds to a particular polypeptide is made by any one of a number of standard immunoassay methods; for

instance, Western blotting (See, e.g., Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

To determine that a given antibody preparation (such as a preparation produced in a mouse against a polypeptide having the amino acid sequence set forth in SEQ ID NO: 2) specifically detects the appropriate polypeptide (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO: 2) by Western blotting, total cellular protein can be extracted from cells and separated by SDS-polyacrylamide gel electrophoresis. The separated total cellular protein can then be transferred to a membrane (e.g., nitrocellulose), and the antibody preparation incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies can be detected using an appropriate secondary antibody (e.g., an anti-mouse antibody) conjugated to an enzyme such as alkaline phosphatase since application of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a densely blue-colored compound by immuno-localized alkaline phosphatase.

Substantially pure polypeptides suitable for use as an immunogen can be obtained from transfected cells, transformed cells, or wild-type cells. Polypeptide concentrations in the final preparation can be adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. In addition, polypeptides ranging in size from full-length polypeptides to polypeptides having as few as nine amino acid residues can be utilized as immunogens. Such polypeptides can be produced in cell culture, can be chemically synthesized using standard methods, or can be obtained by cleaving large polypeptides into smaller polypeptides that can be purified. Polypeptides having as few as nine amino acid residues in length can be immunogenic when presented to an immune system in the context of a Major Histocompatibility Complex (MHC) molecule such as an MHC class I or MHC class II molecule. Accordingly, polypeptides having at least 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 900, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, or more consecutive amino acid residues of any

amino acid sequence disclosed herein can be used as immunogens for producing antibodies.

Monoclonal antibodies to any of the polypeptides disclosed herein can be prepared from murine hybridomas according to the classic method of Kohler & Milstein (Nature 256:495 (1975)) or a derivative method thereof.

Polyclonal antiserum containing antibodies to the heterogeneous epitopes of any polypeptide disclosed herein can be prepared by immunizing suitable animals with the polypeptide (or fragment thereof), which can be unmodified or modified to enhance immunogenicity. An effective immunization protocol for rabbits can be found in Vaitukaitis *et al.* (*J. Clin. Endocrinol. Metab.* 33:988-991 (1971)).

Antibody fragments can be used in place of whole antibodies and can be readily expressed in prokaryotic host cells. Methods of making and using immunologically effective portions of monoclonal antibodies, also referred to as "antibody fragments," are well known and include those described in Better & Horowitz (*Methods Enzymol.* 178:476-496 (1989)), Glockshuber *et al.* (*Biochemistry* 29:1362-1367 (1990)), U.S. Pat. No. 5,648,237 ("Expression of Functional Antibody Fragments"), U.S. Pat. No. 4,946,778 ("Single Polypeptide Chain Binding Molecules"), U.S. Pat. No. 5,455,030 ("Immunotherapy Using Single Chain Polypeptide Binding Molecules"), and references cited therein.

Operably linked: A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence whenever the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two polypeptide-coding regions, in the same reading frame.

Probes and primers: Nucleic acid probes and primers can be prepared readily based on the amino acid sequences and nucleic acid sequences provided herein. A "probe" includes an isolated nucleic acid containing a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed in, for example, Sambrook *et al.* (ed.), *Molecular Cloning:*

A Laboratory Manual 2nd-ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, and Ausubel *et al.* (ed.) Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987.

5 “Primers” are typically nucleic acid molecules having ten or more nucleotides (e.g., nucleic acid molecules having between about 10 nucleotides and about 100 nucleotides). A primer can be annealed to a complementary target nucleic acid strand by nucleic acid hybridization to form a hybrid between the primer and the target nucleic acid strand, and then extended along the target nucleic acid strand by, for example, a DNA
10 polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, for example, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

 Methods for preparing and using probes and primers are described, for example, in references such as Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual,
15 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; Ausubel *et al.* (ed.), Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987; and Innis *et al.*, PCR
 Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR
 primer pairs can be derived from a known sequence, for example, by using computer
20 programs intended for that purpose such as Primer (Version 0.5, .COPYRGT. 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with the
 length, but that a probe or primer can range in size from a full-length sequence to sequences as short as five consecutive nucleotides. Thus, for example, a primer of 20
25 consecutive nucleotides can anneal to a target with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers can be selected that comprise, for example, 10, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550,
30 1600, 1650, 1700, 1750, 1800, 1850, 1900, 2000, 2050, 2100, 2150, 2200, 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2600, 2650, 2700, 2750, 2800, 2850, 2900, 3000, 3050,

3100, 3150, 3200, 3250, 3300, 3350, 3400, 3450, 3500, 3550, 3600, 3650, 3700, 3750, 3800, 3850, 3900, 4000, 4050, 4100, 4150, 4200, 4250, 4300, 4350, 4400, 4450, 4500, 4550, 4600, 4650, 4700, 4750, 4800, 4850, 4900, 5000, 5050, 5100, 5150, 5200, 5250, 5300, 5350, 5400, 5450, or more consecutive nucleotides.

- 5 **Percent sequence identity:** The "percent sequence identity" between a particular nucleic acid or amino acid sequence and a sequence referenced by a particular sequence identification number is determined as follows. First, a nucleic acid or amino acid sequence is compared to the sequence set forth in a particular sequence identification number using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of
- 10 BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained from Fish & Richardson's web site (www.fr.com) or the United States government's National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq
- 15 performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence
- 20 to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to -1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2. To compare two amino acid sequences,
- 25 the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a
- 30 comparison between two amino acid sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the

designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence (e.g., SEQ ID NO:1), or by an articulated length (e.g., 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with the sequence set forth in SEQ ID NO:1 is 75.0 percent identical to the sequence set forth in SEQ ID NO:1 (i.e., $1166 \div 1554 \times 100 = 75.0$). It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 is rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 is rounded up to 75.2. It is also noted that the length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (i.e., $15 \div 20 \times 100 = 75$).

20		1	20
	Target Sequence:	AGGTCGTGTACTGTCAGTCA	
	Identified Sequence:	ACGTGGTGAAGTCCAGTGA	

25 **Conservative substitution:** The term "conservative substitution" as used herein refers to any of the amino acid substitutions set forth in Table 1. Typically, conservative substitutions have little to no impact on the activity of a polypeptide. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard

30 procedures such as site-directed mutagenesis or PCR.

Table 1

Original Residue	Conservative Substitution(s)
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

II. Metabolic Pathways

The invention provides methods and materials related to producing 3-HP as well as other organic compounds (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP). Specifically, the invention provides isolated nucleic acids, polypeptides, host cells, and methods and materials for producing 3-HP as well as other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, polymerized 3-HP, and esters of 3-HP.

Accordingly, the invention provides several metabolic pathways that can be used to produce organic compounds from PEP (Figures 1-5, 43-44, 54, and 55). As depicted in Figure 1, lactate can be converted into lactyl-CoA by a polypeptide having CoA transferase activity (EC 2.8.3.1); the resulting lactyl-CoA can be converted into acrylyl-CoA by a polypeptide (or multiple polypeptide complex such as an activated E2 α and E2 β complex) having lactyl-CoA dehydratase activity (EC 4.2.1.54); the resulting acrylyl-CoA can be converted into 3-hydroxypropionyl-CoA (3-HP-CoA) by a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (EC 4.2.1.-); and the resulting 3-HP-CoA can be converted into 3-HP by a polypeptide having CoA transferase activity, a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), or a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity (EC 3.1.2.4).

Polypeptides having CoA transferase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Megasphaera elsdenii*, *Clostridium propionicum*, *Clostridium kluyveri*, and *Escherichia coli*. For example, nucleic acid that encodes a polypeptide having CoA transferase activity can be obtained from *Megasphaera elsdenii* as described in Example 1 and can have a sequence as set forth in SEQ ID NO: 1. In addition, polypeptides having CoA transferase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 1 provided herein can be used to encode a polypeptide having CoA transferase activity.

Polypeptides (or the polypeptides of a multiple polypeptide complex such as an activated E2 α and E2 β complex) having lactyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Megasphaera elsdenii* and *Clostridium propionicum*. For example, nucleic acid encoding an E1 activator, an E2 α subunit, and an E2 β subunit that can form a multiple polypeptide complex having lactyl-CoA dehydratase activity can be obtained from *Megasphaera elsdenii* as described in Example 2. The nucleic acid encoding the E1 activator can contain a sequence as set forth in SEQ ID NO: 9; the nucleic acid encoding the E2 α subunit can contain a sequence as set forth in SEQ ID NO: 17; and the nucleic acid encoding the E2 β subunit can contain a sequence as set forth in SEQ ID NO: 25. In addition, polypeptides (or the polypeptides of a multiple polypeptide complex) having

lactyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 9, 17, and 25 provided herein can be used to encode the polypeptides of a multiple polypeptide complex having CoA transferase activity.

5 Polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Chloroflexus aurantiacus*, *Candida rugosa*, *Rhodospirillum rubrum*, and *Rhodobacter capsulatus*. For example, nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be obtained from *Chloroflexus*
10 *aurantiacus* as described in Example 3 and can have a sequence as set forth in SEQ ID NO: 40. In addition, polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 40 provided herein can be used to encode a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity.

15 Polypeptides having 3-hydroxypropionyl-CoA hydrolase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Candida rugosa*. Polypeptides having 3-hydroxyisobutryl-CoA hydrolase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Pseudomonas fluorescens*, *rattus*, and
20 *homo sapiens*. For example, nucleic acid that encodes a polypeptide having 3-hydroxyisobutryl-CoA hydrolase activity can be obtained from *homo sapiens* and can have a sequence as set forth in GenBank® accession number U66669.

The term "polypeptide having enzymatic activity" as used herein refers to any polypeptide that catalyzes a chemical reaction of other substances without itself being
25 destroyed or altered upon completion of the reaction. Typically, a polypeptide having enzymatic activity catalyzes the formation of one or more products from one or more substrates. Such polypeptides can have any type of enzymatic activity including, without limitation, the enzymatic activity or enzymatic activities associated with enzymes such as dehydratases/hydratases, 3-hydroxypropionyl-CoA dehydratases/hydratases, CoA
30 transferases, lactyl-CoA dehydratases, 3-hydroxypropionyl-CoA hydrolases, 3-

hydroxyisobutryl-CoA hydrolases, poly hydroxyacid synthases, CoA synthetases, malonyl-CoA reductases, β -alanine ammonia lyases, and lipases.

As depicted in Figure 2, lactate can be converted into lactyl-CoA by a polypeptide having CoA synthetase activity (EC 6.2.1.-); the resulting lactyl-CoA can be converted
5 into acrylyl-CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can be converted into 3-HP-CoA by a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity; and the resulting 3-HP-CoA can be converted into polymerized 3-HP by a polypeptide having poly hydroxyacid synthase activity (EC 2.3.1.-). Polypeptides having CoA synthetase activity
10 as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Escherichia coli*, *Rhodobacter sphaeroides*, *Saccharomyces cerevisiae*, and *Salmonella enterica*. For example, nucleic acid that encodes a polypeptide having CoA synthetase activity can be obtained from *Escherichia coli* and can have a sequence as set forth in GenBank® accession number U00006. Polypeptides (or multiple
15 polypeptide complexes) having lactyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides can be obtained as provided herein. Polypeptides having 3-hydroxypropionyl-CoA dehydratase activity as well as nucleic acid encoding such polypeptides also can be obtained as provided herein. Polypeptides having poly hydroxyacid synthase activity as well as nucleic acid encoding such polypeptides can be
20 obtained from various species including, without limitation, *Rhodobacter sphaeroides*, *Comamonas acidovorans*, *Ralstonia eutropha*, and *Pseudomonas oleovorans*. For example, nucleic acid that encodes a polypeptide having poly hydroxyacid synthase activity can be obtained from *Rhodobacter sphaeroides* and can have a sequence as set forth in GenBank® accession number X97200.

25 As depicted in Figure 3, lactate can be converted into lactyl-CoA by a polypeptide having CoA transferase activity; the resulting lactyl-CoA can be converted into acrylyl-CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can be converted into 3-HP-CoA by a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity; the resulting 3-HP-CoA can be
30 converted into 3-HP by a polypeptide having CoA transferase activity, a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity, or a polypeptide having 3-

hydroxyisobutryl-CoA hydrolase activity; and the resulting 3-HP can be converted into an ester of 3-HP by a polypeptide having lipase activity (EC 3.1.1.-). Polypeptides having lipase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Candida rugosa*, *Candida tropicalis*, and
5 *Candida albicans*. For example, nucleic acid that encodes a polypeptide having lipase activity can be obtained from *Candida rugosa* and can have a sequence as set forth in GenBank® accession number A81171.

As depicted in Figure 4, lactate can be converted into lactyl-CoA by a polypeptide having CoA synthetase activity; the resulting lactyl-CoA can be converted into acrylyl-
10 CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; and the resulting acrylyl-CoA can be converted into polymerized acrylate by a polypeptide having poly hydroxyacid synthase activity.

As depicted in Figure 5, lactate can be converted into lactyl-CoA by a polypeptide having CoA transferase activity; the resulting lactyl-CoA can be converted into acrylyl-
15 CoA by a polypeptide (or multiple polypeptide complex) having lactyl-CoA dehydratase activity; the resulting acrylyl-CoA can be converted into acrylate by a polypeptide having CoA transferase activity; and the resulting acrylate can be converted into an ester of acrylate by a polypeptide having lipase activity.

As depicted in Figure 44, acetyl-CoA can be converted into malonyl-CoA by a
20 polypeptide having acetyl-CoA carboxylase activity, and the resulting malonyl-CoA can be converted into 3-HP by a polypeptide having malonyl-CoA reductase activity. Polypeptides having acetyl-CoA carboxylase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Escherichia coli* and *Chloroflexus aurantiacus*. For example, nucleic acid that encodes a
25 polypeptide having acetyl-CoA carboxylase activity can be obtained from *Escherichia coli* and can have a sequence as set forth in GenBank® accession number M96394 or U18997. Polypeptides having malonyl-CoA reductase activity as well as nucleic acid encoding such polypeptides can be obtained from various species including, without limitation, *Chloroflexus aurantiacus*, *Sulfolobus metacillus*, and *Acidianus brierleyi*. For
30 example, nucleic acid that encodes a polypeptide having malonyl-CoA reductase activity can be obtained as described herein and can have a sequence similar to the sequence set

forth in SEQ ID NO: 140. In addition, polypeptides having malonyl-CoA reductase activity as well as nucleic acid encoding such polypeptides can be obtained as described herein. For example, the variations to SEQ ID NO: 140 provided herein can be used to encode a polypeptide having malonyl-CoA reductase activity.

5 Polypeptides having malonyl-CoA reductase activity can use NADPH as a co-factor. For example, the polypeptide having the amino acid sequence set forth in SEQ ID NO: 141 is a polypeptide having malonyl-CoA reductase activity that uses NADPH as a co-factor when converting malonyl-CoA into 3-HP. Likewise, polypeptides having malonyl-CoA reductase activity can use NADH as a co-factor. Such polypeptides can be
10 obtained by converting a polypeptide that has malonyl-CoA reductase activity and uses NADPH as a cofactor into a polypeptide that has malonyl-CoA reductase activity and uses NADH as a cofactor. Any method can be used to convert a polypeptide that uses NADPH as a cofactor into a polypeptide that uses NADH as a cofactor such as those described by others (Eppink *et al.*, *J. Mol. Biol.*, 292(1):87-96 (1999), Hall and Tomsett,
15 *Microbiology*, 146(Pt 6):1399-406 (2000); and Dohr *et al.*, *Proc. Natl. Acad. Sci.*, 98(1):81-86 (2001)). For example, mutagenesis can be used to convert the polypeptide encoded by the nucleic acid sequence set forth in SEQ ID NO: 140 into a polypeptide that, when converting malonyl-CoA into 3-HP, uses NADH as a co-factor instead of NADPH.

20 As depicted in Figure 43, propionate can be converted into propionyl-CoA by a polypeptide having CoA synthetase activity such as the polypeptide having the sequence set forth in SEQ ID NO: 39; the resulting propionyl-CoA can be converted into acrylyl-CoA by a polypeptide having dehydrogenase activity such as the polypeptide having the sequence set forth in SEQ ID NO: 39; and the resulting acrylyl-CoA can be converted
25 into (1) acrylate by a polypeptide having CoA transferase activity or CoA hydrolase activity, (2) 3-HP-CoA by a polypeptide having 3-HP dehydratase activity (also referred to as acrylyl-CoA hydratase or simply hydratase) such as the polypeptide having the sequence set forth in SEQ ID NO:39, or (3) polymerized acrylate by a polypeptide having poly hydroxyacid synthase activity. The resulting acrylate can be converted into an ester
30 of acrylate by a polypeptide having lipase activity. The resulting 3-HP-CoA can be converted into (1) 3-HP by a polypeptide having CoA transferase activity, a polypeptide

having 3-hydroxypropionyl-CoA hydrolase activity (EC 3.1.2.-), or a polypeptide having 3-hydroxyisobutyryl-CoA hydrolase activity (EC 3.1.2.4), or (2) polymerized 3-HP by a polypeptide having poly hydroxyacid synthase activity (EC 2.3.1.-).

As depicted in Figure 54, PEP can be converted into β -alanine. β -alanine can be converted into β -alanyl-CoA through the use of a polypeptide having CoA transferase activity. β -alanyl-CoA can then be converted into acrylyl-CoA through the use of a polypeptide having β -alanyl-CoA ammonia lyase activity. Acrylyl-CoA can then be converted into 3-HP-CoA through the use of a polypeptide having 3-HP-CoA dehydratase activity, and a polypeptide having glutamate dehydrogenase activity can be used to convert 3-HP-CoA into 3-HP.

As depicted in Figure 55, 3-HP can be made from β -alanine by first contacting β -alanine with a polypeptide having 4,4-aminobutyrate aminotransferase activity to create malonate semialdehyde. The malonate semialdehyde can be converted into 3-HP with a polypeptide having 3-HP dehydrogenase activity or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

III. Nucleic acid molecules and polypeptides

The invention provides isolated nucleic acid that contains the entire nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. In addition, the invention provides isolated nucleic acid that contains a portion of the nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. For example, the invention provides isolated nucleic acid that contains a 15 nucleotide sequence identical to any 15-nucleotide sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163 including, without limitation, the sequence starting at nucleotide number 1 and ending at nucleotide number 15, the sequence starting at nucleotide number 2 and ending at nucleotide number 16, the sequence starting at nucleotide number 3 and ending at nucleotide number 17, and so forth. It will be appreciated that the invention also provides isolated nucleic acid that contains a nucleotide sequence that is greater than 15 nucleotides (e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more nucleotides) in length and identical to any portion of the sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129,

140, 142, 162, or 163. For example, the invention provides isolated nucleic acid that contains a 25 nucleotide sequence identical to any 25 nucleotide sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163 including, without limitation, the sequence starting at nucleotide number 1 and ending at nucleotide number 25, the sequence starting at nucleotide number 2 and ending at nucleotide number 26, the sequence starting at nucleotide number 3 and ending at nucleotide number 27, and so forth. Additional examples include, without limitation, isolated nucleic acids that contain a nucleotide sequence that is 50 or more nucleotides (e.g., 100, 150, 200, 250, 300, or more nucleotides) in length and identical to any portion of the sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. Such isolated nucleic acids can include, without limitation, those isolated nucleic acids containing a nucleic acid sequence represented in a single line of sequence depicted in Figure 6, 10, 14, 18, 22, 23, 25, 27, 29, 31, 39, 49, or 51 since each line of sequence depicted in these figures, with the possible exception of the last line, provides a nucleotide sequence containing at least 50 bases.

In addition, the invention provides isolated nucleic acid that contains a variation of the nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. For example, the invention provides isolated nucleic acid containing a nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163 that contains a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such isolated nucleic acid molecules can share at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163.

The invention provides multiple examples of isolated nucleic acid that contains a variation of a nucleic acid sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. For example, Figure 8 provides the sequence set forth in SEQ ID NO:1 aligned with three other nucleic acid sequences. Examples of variations of the sequence set forth in SEQ ID NO:1 include, without limitation, any variation of the sequence set forth in SEQ ID NO:1 provided in Figure 8. Such variations are provided in

Figure 8 in that a comparison of the nucleotide (or lack thereof) at a particular position of the sequence set forth in SEQ ID NO:1 with the nucleotide (or lack thereof) at the same aligned position of any of the other three nucleic acid sequences depicted in Figure 8 (i.e., SEQ ID NOs:3, 4, and 5) provides a list of specific changes for the sequence set forth in SEQ ID NO:1. For example, the "a" at position 49 of SEQ ID NO:1 can be substituted with an "c" as indicated in Figure 8. As also indicated in Figure 8, the "a" at position 590 of SEQ ID NO:1 can be substituted with a "atgg"; an "aaac" can be inserted before the "g" at position 393 of SEQ ID NO:1; or the "gaa" at position 736 of SEQ ID NO:1 can be deleted. It will be appreciated that the sequence set forth in SEQ ID NO:1 can contain any number of variations as well as any combination of types of variations. For example, the sequence set forth in SEQ ID NO:1 can contain one variation provided in Figure 8 or more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, or more) of the variations provided in Figure 8. It is noted that the nucleic acid sequences provided by Figure 8 can encode polypeptides having CoA transferase activity. The invention also provides isolated nucleic acid that contains a variant of a portion of the sequence set forth in SEQ ID NO:1 as depicted in Figure 8 and described herein.

Likewise, Figure 12 provides variations of SEQ ID NO:9 and portions thereof; Figure 16 provides variations of SEQ ID NO:17 and portions thereof; Figure 20 provides variations of SEQ ID NO:25 and portions thereof; Figure 32 provides variations of SEQ ID NO:40 and portions thereof; and Figure 53 provides variations of SEQ ID NO:140.

The invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes the entire amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. In addition, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes a portion of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes a 15 amino acid sequence identical to any 15 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 15, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 16, the sequence starting at amino acid residue number 3 and ending

at amino acid residue number 17, and so forth. It will be appreciated that the invention also provides isolated nucleic acid that contains a nucleic acid sequence that encodes an amino acid sequence that is greater than 15 amino acid residues (e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes a 25 amino acid sequence identical to any 25 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 25, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 26, the sequence starting at amino acid residue number 3 and ending at amino acid residue number 27, and so forth. Additional examples include, without limitation, isolated nucleic acids that contain a nucleic acid sequence that encodes an amino acid sequence that is 50 or more amino acid residues (e.g., 100, 150, 200, 250, 300, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. Such isolated nucleic acids can include, without limitation, those isolated nucleic acids containing a nucleic acid sequence that encodes an amino acid sequence represented in a single line of sequence depicted in Figure 7, 11, 15, 19, 24, 26, 28, 30, or 50 since each line of sequence depicted in these figures, with the possible exception of the last line, provides an amino acid sequence containing at least 50 residues.

In addition, the invention provides isolated nucleic acid that contains a nucleic acid sequence that encodes an amino acid sequence having a variation of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides isolated nucleic acid containing a nucleic acid sequence encoding an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 that contains a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such isolated nucleic acid molecules can contain a nucleic acid sequence encoding an amino acid sequence that shares at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99 percent sequence identity with a

sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161.

The invention provides multiple examples of isolated nucleic acid containing a nucleic acid sequence encoding an amino acid sequence having a variation of an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, Figure 9 provides the amino acid sequence set forth in SEQ ID NO:2 aligned with three other amino acid sequences. Examples of variations of the sequence set forth in SEQ ID NO:2 include, without limitation, any variation of the sequence set forth in SEQ ID NO:2 provided in Figure 9. Such variations are provided in Figure 9 in that a comparison of the amino acid residue (or lack thereof) at a particular position of the sequence set forth in SEQ ID NO:2 with the amino acid residue (or lack thereof) at the same aligned position of any of the other three amino acid sequences of Figure 9 (i.e., SEQ ID NOs:6, 7, and 8) provides a list of specific changes for the sequence set forth in SEQ ID NO:2. For example, the "k" at position 17 of SEQ ID NO:2 can be substituted with a "p" or "h" as indicated in Figure 9. As also indicated in Figure 9, the "v" at position 125 of SEQ ID NO:2 can be substituted with an "i" or "f". It will be appreciated that the sequence set forth in SEQ ID NO:2 can contain any number of variations as well as any combination of types of variations. For example, the sequence set forth in SEQ ID NO:2 can contain one variation provided in Figure 9 or more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, or more) of the variations provided in Figure 9. It is noted that the amino acid sequences provided in Figure 9 can be polypeptides having CoA transferase activity.

The invention also provides isolated nucleic acid containing a nucleic acid sequence encoding an amino acid sequence that contains a variant of a portion of the sequence set forth in SEQ ID NO:2 as depicted in Figure 9 and described herein.

Likewise, Figure 13 provides variations of SEQ ID NO:10 and portions thereof; Figure 17 provides variations of SEQ ID NO:18 and portions thereof; Figure 21 provides variations of SEQ ID NO:26 and portions thereof; Figure 33 provides variations of SEQ ID NO:41 and portions thereof; Figures 40, 41, and 42 provide variations of SEQ ID NO:39; and Figure 52 provides variations of SEQ ID NO:141 and portions thereof.

It is noted that codon preferences and codon usage tables for a particular species can be used to engineer isolated nucleic acid molecules that take advantage of the codon

usage preferences of that particular species. For example, the isolated nucleic acid provided herein can be designed to have codons that are preferentially used by a particular organism of interest.

The invention also provides isolated nucleic acid that is at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000 bases in length) and hybridizes, under hybridization conditions, to the sense or antisense strand of a nucleic acid having the sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163. The hybridization conditions can be moderately or highly stringent hybridization conditions.

The invention provides polypeptides that contain the entire amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. In addition, the invention provides polypeptides that contain a portion of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides polypeptides that contain a 15 amino acid sequence identical to any 15 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 15, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 16, the sequence starting at amino acid residue number 3 and ending at amino acid residue number 17, and so forth. It will be appreciated that the invention also provides polypeptides that contain an amino acid sequence that is greater than 15 amino acid residues (e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, the invention provides polypeptides that contain a 25 amino acid sequence identical to any 25 amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 including, without limitation, the sequence starting at amino acid residue number 1 and ending at amino acid residue number 25, the sequence starting at amino acid residue number 2 and ending at amino acid residue number 26, the sequence starting at amino acid residue number 3 and ending at amino acid residue number 27, and so forth. Additional examples include, without limitation, polypeptides

that contain an amino acid sequence that is 50 or more amino acid residues (e.g., 100, 150, 200, 250, 300, or more amino acid residues) in length and identical to any portion of the sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. Such polypeptides can include, without limitation, those polypeptides containing a amino acid
5 sequence represented in a single line of sequence depicted in Figure 7, 11, 15, 19, 24, 26, 28, 30, or 50 since each line of sequence depicted in these figures, with the possible exception of the last line, provides an amino acid sequence containing at least 50 residues.

In addition, the invention provides polypeptides that an amino acid sequence having a variation of the amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35,
10 37, 39, 41, 141, 160, or 161. For example, the invention provides polypeptides containing an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 that contains a single insertion, a single deletion, a single substitution, multiple insertions, multiple deletions, multiple substitutions, or any combination thereof (e.g., single deletion together with multiple insertions). Such polypeptides can contain an
15 amino acid sequence that shares at least 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99 percent sequence identity with a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161.

The invention provides multiple examples of polypeptides containing an amino acid sequence having a variation of an amino acid sequence set forth in SEQ ID NO:2,
20 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161. For example, Figure 9 provides the amino acid sequence set forth in SEQ ID NO:2 aligned with three other amino acid sequences. Examples of variations of the sequence set forth in SEQ ID NO:2 include, without limitation, any variation of the sequence set forth in SEQ ID NO:2 provided in Figure 9. Such variations are provided in Figure 9 in that a comparison of the amino acid residue
25 (or lack thereof) at a particular position of the sequence set forth in SEQ ID NO:2 with the amino acid residue (or lack thereof) at the same aligned position of any of the other three amino acid sequences of Figure 9 (i.e., SEQ ID NOs:6, 7, and 8) provides a list of specific changes for the sequence set forth in SEQ ID NO:2. For example, the "k" at position 17 of SEQ ID NO:2 can be substituted with a "p" or "h" as indicated in Figure 9.
30 As also indicated in Figure 9, the "v" at position 125 of SEQ ID NO:2 can be substituted with an "i" or "f". It will be appreciated that the sequence set forth in SEQ ID NO:2 can

contain any number of variations as well as any combination of types of variations. For example, the sequence set forth in SEQ ID NO:2 can contain one variation provided in Figure 9 or more than one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, or more) of the variations provided in Figure 9. It is noted that the amino acid sequences provided in Figure 9 can be polypeptides having CoA transferase activity.

The invention also provides polypeptides containing an amino acid sequence that contains a variant of a portion of the sequence set forth in SEQ ID NO:2 as depicted in Figure 9 and described herein.

Likewise, Figure 13 provides variations of SEQ ID NO:10 and portions thereof; Figure 17 provides variations of SEQ ID NO:18 and portions thereof; Figure 21 provides variations of SEQ ID NO:26 and portions thereof; Figure 33 provides variations of SEQ ID NO:41 and portions thereof, Figures 40, 41, and 42 provide variations of SEQ ID NO:39; and Figure 52 provides variations of SEQ ID NO:141 and portions thereof.

Polypeptides having a variant amino acid sequence can retain enzymatic activity. Such polypeptides can be produced by manipulating the nucleotide sequence encoding a polypeptide using standard procedures such as site-directed mutagenesis or PCR. One type of modification includes the substitution of one or more amino acid residues for amino acid residues having a similar biochemical property. For example, a polypeptide can have an amino acid sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 with one or more conservative substitutions.

More substantial changes can be obtained by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining: (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation; (b) the charge or hydrophobicity of the polypeptide at the target site; or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in polypeptide function are those in which: (a) a hydrophilic residue, e.g., serine or threonine, is substituted for (or by) a hydrophobic residue, e.g., leucine, isoleucine, phenylalanine, valine or alanine; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysine, arginine, or histidine, is substituted for (or by) an electronegative residue, e.g., glutamic acid or aspartic acid; or

(d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. The effects of these amino acid substitutions (or other deletions or additions) can be assessed for polypeptides having enzymatic activity by analyzing the ability of the polypeptide to catalyze the conversion of the same
5 substrate as the related native polypeptide to the same product as the related native polypeptide. Accordingly, polypeptides having 5, 10, 20, 30, 40, 50 or less conservative substitutions are provided by the invention.

Polypeptides and nucleic acid encoding polypeptide can be produced by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these
10 techniques are provided in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual* 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring, Harbor, N.Y., 1989, Ch. 15. Nucleic acid molecules can contain changes of a coding region to fit the codon usage bias of the particular organism into which the molecule is to be introduced.

15 Alternatively, the coding region can be altered by taking advantage of the degeneracy of the genetic code to alter the coding sequence in such a way that, while the nucleic acid sequence is substantially altered, it nevertheless encodes a polypeptide having an amino acid sequence identical or substantially similar to the native amino acid sequence. For example, the ninth amino acid residue of the sequence set forth in SEQ ID
20 NO: 2 is alanine, which is encoded in the open reading frame by the nucleotide codon triplet GCT. Because of the degeneracy of the genetic code, three other nucleotide codon triplets—GCA, GCC, and GCG—also code for alanine. Thus, the nucleic acid sequence of the open reading frame can be changed at this position to any of these three codons without affecting the amino acid sequence of the encoded polypeptide or the
25 characteristics of the polypeptide. Based upon the degeneracy of the genetic code, nucleic acid variants can be derived from a nucleic acid sequence disclosed herein using a standard DNA mutagenesis techniques as described herein, or by synthesis of nucleic acid sequences. Thus, this invention also encompasses nucleic acid molecules that encode the same polypeptide but vary in nucleic acid sequence by virtue of the degeneracy of the
30 genetic code.

IV. Methods of Making 3-HP and Other Organic Acids

Each step provided in the pathways depicted in Figures 1-5, 43-44, 54, and 55 can be performed within a cell (*in vivo*) or outside a cell (*in vitro*, e.g., in a container or column). Additionally, the organic acid products can be generated through a combination of *in vivo* synthesis and *in vitro* synthesis. Moreover, the *in vitro* synthesis step, or steps, can be via chemical reaction or enzymatic reaction.

For example, a microorganism provided herein can be used to perform the steps provided in Figure 1, or an extract containing polypeptides having the indicated enzymatic activities can be used to perform the steps provided in Figure 1. In addition, chemical treatments can be used to perform the conversions provided in Figures 1-5, 43-44, 54, and 55. For example, acrylyl-CoA can be converted into acrylate by hydrolysis. Other chemical treatments include, without limitation, trans esterification to convert acrylate into an acrylate ester.

Carbon sources suitable as starting points for bioconversion include carbohydrates and synthetic intermediates. Examples of carbohydrates which cells are capable of metabolizing to pyruvate include sugars such as dextrose, triglycerides, and fatty acids.

Additionally, intermediate chemical products can be starting points. For example, acetic acid and carbon dioxide can be introduced into a fermentation broth. Acetyl-CoA, malonyl-CoA, and 3-HP can be sequentially produced using a polypeptide having CoA synthase activity, a polypeptide having acetyl-CoA carboxylase activity, and a polypeptide having malonyl-CoA reductase activity. Other useful intermediate chemical starting points can include propionic acid, acrylic acid, lactic acid, pyruvic acid, and β -alanine.

A. Expression of Polypeptides

The polypeptides described herein can be produced individually in a host cell or in combination in a host cell. Moreover, the polypeptides having a particular enzymatic activity can be a polypeptide that is either naturally-occurring or non-naturally-occurring. A naturally-occurring polypeptide is any polypeptide having an amino acid sequence as found in nature, including wild-type and polymorphic polypeptides. Such naturally-occurring polypeptides can be obtained from any species including, without limitation,

animal (e.g., mammalian), plant, fungal, and bacterial species. A non-naturally-occurring polypeptide is any polypeptide having an amino acid sequence that is not found in nature. Thus, a non-naturally-occurring polypeptide can be a mutated version of a naturally-occurring polypeptide, or an engineered polypeptide. For example, a non-naturally-occurring polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be a mutated version of a naturally-occurring polypeptide having 3-hydroxypropionyl-CoA dehydratase activity that retains at least some 3-hydroxypropionyl-CoA dehydratase activity. A polypeptide can be mutated by, for example, sequence additions, deletions, substitutions, or combinations thereof.

The invention provides genetically modified cells that can be used to perform one or more steps of the steps in the metabolic pathways described herein or the genetically modified cells can be used to produce the disclosed polypeptides for subsequent use *in vitro*. For example, an individual microorganism can contain exogenous nucleic acid such that each of the polypeptides necessary to perform the steps depicted in Figures 1, 2, 3, 4, 5, 43, 44, 54, or 55 are expressed. It is important to note that such cells can contain any number of exogenous nucleic acid molecules. For example, a particular cell can contain six exogenous nucleic acid molecules with each one encoding one of the six polypeptides necessary to convert lactate into 3-HP as depicted in Figure 1, or a particular cell can endogenously produce polypeptides necessary to convert lactate into acrylyl-CoA while containing exogenous nucleic acid that encodes polypeptides necessary to convert acrylyl-CoA into 3-HP.

In addition, a single exogenous nucleic acid molecule can encode one or more than one polypeptide. For example, a single exogenous nucleic acid molecule can contain sequences that encode three different polypeptides. Further, the cells described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid molecule. For example, a particular cell can contain about 50 copies of the constructs depicted in Figure 34, 35, 36, 37, 38, or 45. Again, the cells described herein can contain more than one particular exogenous nucleic acid molecule. For example, a particular cell can contain about 50 copies of exogenous nucleic acid molecule X as well as about 75 copies of exogenous nucleic acid molecule Y.

In another embodiment, a cell within the scope of the invention can contain an exogenous nucleic acid molecule that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. Such cells can have any level of 3-hydroxypropionyl-CoA dehydratase activity. For example, a cell containing an exogenous nucleic acid molecule that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can have 3-hydroxypropionyl-CoA dehydratase activity with a specific activity greater than about 1 mg 3-HP-CoA formed per gram dry cell weight per hour (e.g., greater than about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, or more mg 3-HP-CoA formed per gram dry cell weight per hour). Alternatively, a cell can have 3-hydroxypropionyl-CoA dehydratase activity such that a cell extract from 1×10^6 cells has a specific activity greater than about 1 μ g 3-HP-CoA formed per mg total protein per 10 minutes (e.g., greater than about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 200, 250, 300, 350, 400, 500, or more μ g 3-HP-CoA formed per mg total protein per 10 minutes).

A nucleic acid molecule encoding a polypeptide having enzymatic activity can be identified and obtained using any method such as those described herein. For example, nucleic acid molecules that encode a polypeptide having enzymatic activity can be identified and obtained using common molecular cloning or chemical nucleic acid synthesis procedures and techniques, including PCR. In addition, standard nucleic acid sequencing techniques and software programs that translate nucleic acid sequences into amino acid sequences based on the genetic code can be used to determine whether or not a particular nucleic acid has any sequence homology with known enzymatic polypeptides. Sequence alignment software such as MEGALIGN[®] (DNASTAR, Madison, WI, 1997) can be used to compare various sequences. In addition, nucleic acid molecules encoding known enzymatic polypeptides can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and base substitutions, as well as combinations of deletions, insertions, and base substitutions. Further, nucleic acid and amino acid databases (e.g., GenBank[®]) can be used to identify a nucleic acid sequence that encodes a polypeptide having enzymatic activity. Briefly, any amino acid sequence having some homology to a polypeptide having enzymatic activity, or any nucleic acid sequence

having some homology to a sequence encoding a polypeptide having enzymatic activity can be used as a query to search GenBank®. The identified polypeptides then can be analyzed to determine whether or not they exhibit enzymatic activity.

In addition, nucleic acid hybridization techniques can be used to identify and
5 obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. Briefly, any nucleic acid molecule that encodes a known enzymatic polypeptide, or fragment thereof, can be used as a probe to identify a similar nucleic acid molecules by hybridization under conditions of moderate to high stringency. Such similar nucleic acid molecules then can be isolated, sequenced, and analyzed to determine whether the
10 encoded polypeptide has enzymatic activity.

Expression cloning techniques also can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. For example, a substrate known to interact with a particular enzymatic polypeptide can be used to screen
15 a phage display library containing that enzymatic polypeptide. Phage display libraries can be generated as described elsewhere (Burritt *et al.*, *Anal. Biochem.* 238:1-13 (1990)), or can be obtained from commercial suppliers such as Novagen (Madison, WI).

Further, polypeptide sequencing techniques can be used to identify and obtain a nucleic acid molecule that encodes a polypeptide having enzymatic activity. For
20 ~~example, a purified polypeptide can be separated by gel electrophoresis, and its amino~~ acid sequence determined by, for example, amino acid microsequencing techniques. Once determined, the amino acid sequence can be used to design degenerate oligonucleotide primers. Degenerate oligonucleotide primers can be used to obtain the nucleic acid encoding the polypeptide by PCR. Once obtained, the nucleic acid can be sequenced, cloned into an appropriate expression vector, and introduced into a
25 microorganism.

Any method can be used to introduce an exogenous nucleic acid molecule into a cell. In fact, many methods for introducing nucleic acid into microorganisms such as bacteria and yeast are well known to those skilled in the art. For example, heat shock, lipofection, electroporation, conjugation, fusion of protoplasts, and biolistic delivery are
30 common methods for introducing nucleic acid into bacteria and yeast cells. See, e.g., Ito

et al., *J. Bacterol.* 153:163-168 (1983); Durrens *et al.*, *Curr. Genet.* 18:7-12 (1990); and Becker and Guarente, *Methods in Enzymology* 194:182-187 (1991).

An exogenous nucleic acid molecule contained within a particular cell of the invention can be maintained within that cell in any form. For example, exogenous nucleic acid molecules can be integrated into the genome of the cell or maintained in an episomal state. In other words, a cell of the invention can be a stable or transient transformant. Again, a microorganism described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid molecule as described herein.

Methods for expressing an amino acid sequence from an exogenous nucleic acid molecule are well known to those skilled in the art. Such methods include, without limitation, constructing a nucleic acid such that a regulatory element promotes the expression of a nucleic acid sequence that encodes a polypeptide. Typically, regulatory elements are DNA sequences that regulate the expression of other DNA sequences at the level of transcription. Thus, regulatory elements include, without limitation, promoters, enhancers, and the like. Any type of promoter can be used to express an amino acid sequence from an exogenous nucleic acid molecule. Examples of promoters include, without limitation, constitutive promoters, tissue-specific promoters, and promoters responsive or unresponsive to a particular stimulus (e.g., light, oxygen, chemical concentration, and the like). Moreover, methods for expressing a polypeptide from an exogenous nucleic acid molecule in cells such as bacterial cells and yeast cells are well known to those skilled in the art. For example, nucleic acid constructs that are capable of expressing exogenous polypeptides within *E. coli* are well known. See, e.g., Sambrook *et al.*, *Molecular cloning: a laboratory manual*, Cold Spring Harbour Laboratory Press, New York, USA, second edition (1989).

B. Production of Organic Acids and Related Products via Host Cells

The nucleic acid and amino acid sequences provided herein can be used with cells to produce 3-HP and/or other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP. Such cells can be from any species including those listed within the taxonomy web pages at the

National Institute of Health sponsored by the United States government (www.ncbi.nlm.nih.gov). The cells can be eukaryotic or prokaryotic. For example, genetically modified cells of the invention can be mammalian cells (e.g., human, murine, and bovine cells), plant cells (e.g., corn, wheat, rice, and soybean cells), fungal cells (e.g., *Aspergillus* and *Rhizopus* cells), yeast cells, or bacterial cells (e.g., *Lactobacillus*, *Lactococcus*, *Bacillus*, *Escherichia*, and *Clostridium* cells). A cell of the invention also can be a microorganism. The term "microorganism" as used herein refers to any microscopic organism including, without limitation, bacteria, algae, fungi, and protozoa. Thus, *E. coli*, *S. cerevisiae*, *Kluveromyces lactis*, *Candida blankii*, *Candida rugosa*, and *Pichia pastoris* are considered microorganisms and can be used as described herein.

Typically, a cell of the invention is genetically modified such that a particular organic compound is produced. In one embodiment, the invention provides cells that make 3-HP from PEP. Examples biosynthetic pathways that can be used by cells to make 3-HP are shown in Figures 1-5, 43-44, 54, and 55.

Generally, cells that are genetically modified to synthesize a particular organic compound contain one or more exogenous nucleic acid molecules that encode polypeptides having specific enzymatic activities. For example, a microorganism can contain exogenous nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity. In this case, acrylyl-CoA can be converted into 3-hydroxypropionic acid-CoA which can lead to the production of 3-HP. It is noted that a cell can be given an exogenous nucleic acid molecule that encodes a polypeptide having an enzymatic activity that catalyzes the production of a compound not normally produced by that cell. Alternatively, a cell can be given an exogenous nucleic acid molecule that encodes a polypeptide having an enzymatic activity that catalyzes the production of a compound that is normally produced by that cell. In this case, the genetically modified cell can produce more of the compound, or can produce the compound more efficiently, than a similar cell not having the genetic modification.

In one embodiment, the invention provides a cell containing an exogenous nucleic acid molecule that encodes a polypeptide having enzymatic activity that leads to the formation of 3-HP. It is noted that the produced 3-HP can be secreted from the cell, eliminating the need to disrupt cell membranes to retrieve the organic compound.

Typically, the cell of the invention produces 3-HP with the concentration being at least about 100 mg per L (e.g., at least about 1 g/L, 5 g/L, 10 g/L, 25 g/L, 50 g/L, 75 g/L, 80 g/L, 90 g/L, 100 g/L, or 120 g/L). When determining the yield of an organic compound such as 3-HP for a particular cell, any method can be used. See, e.g., *Applied*

- 5 *Environmental Microbiology* 59(12):4261-4265 (1993). Typically, a cell within the scope of the invention such as a microorganism catabolizes a hexose carbon source such as glucose. A cell, however, can catabolize a variety of carbon sources such as pentose sugars (e.g., ribose, arabinose, xylose, and lyxose), fatty acids, acetate, or glycerols. In other words, a cell within the scope of the invention can utilize a variety of carbon
10 sources.

- As described herein, a cell within the scope of the invention can contain an exogenous nucleic acid molecule that encodes a polypeptide having enzymatic activity that leads to the formation of 3-HP or other organic compounds such as 1,3-propanediol, acrylic acid, poly-acrylate, acrylate-esters, 3-HP-esters, and poly-3-HP. Methods of
15 identifying cells that contain exogenous nucleic acid are well known to those skilled in the art. Such methods include, without limitation, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis (see hybridization described herein). In some cases, immunohisto-chemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of the
20 polypeptide encoded by that particular nucleic acid molecule. For example, an antibody having specificity for a polypeptide can be used to determine whether or not a particular cell contains nucleic acid encoding that polypeptide. Further, biochemical techniques can be used to determine if a cell contains a particular nucleic acid molecule encoding a polypeptide having enzymatic activity by detecting an organic product produced as a
25 result of the expression of the polypeptide having enzymatic activity. For example, detection of 3-HP after introduction of exogenous nucleic acid that encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity into a cell that does not normally express such a polypeptide can indicate that that cell not only contains the introduced exogenous nucleic acid molecule but also expresses the encoded polypeptide from that
30 introduced exogenous nucleic acid molecule. Methods for detecting specific enzymatic activities or the presence of particular organic products are well known to those skilled in

the art. For example, the presence of an organic compound such as 3-HP can be determined as described elsewhere. See, Sullivan and Clarke, *J. Assoc. Offic. Agr. Chemists*, 38:514-518 (1955).

5 **C. Cells with Reduced Polypeptide Activity**

The invention also provides genetically modified cells having reduced polypeptide activity. The term "reduced" as used herein with respect to a cell and a particular polypeptide's activity refers to a lower level of activity than that measured in a comparable cell of the same species. For example, a particular microorganism lacking
10 enzymatic activity X is considered to have reduced enzymatic activity X if a comparable microorganism has at least some enzymatic activity X. It is noted that a cell can have the activity of any type of polypeptide reduced including, without limitation, enzymes, transcription factors, transporters, receptors, signal molecules, and the like. For example, a cell can contain an exogenous nucleic acid molecule that disrupts a regulatory and/or
15 coding sequence of a polypeptide having pyruvate decarboxylase activity or alcohol dehydrogenase activity. Disrupting pyruvate decarboxylase and/or alcohol dehydrogenase expression can lead to the accumulation of lactate as well as products produced from lactate such as 3-HP, 1,3-propanediol, acrylic acid, poly-acrylate, acrylate-esters, 3-HP-esters, and poly-3-HP. It is also noted that reduced polypeptide activities
20 can be the result of lower polypeptide concentration, lower specific activity of a polypeptide, or combinations thereof. Many different methods can be used to make a cell having reduced polypeptide activity. For example, a cell can be engineered to have a disrupted regulatory sequence or polypeptide-encoding sequence using common mutagenesis or knock-out technology. See, e.g., *Methods in Yeast Genetics* (1997
25 edition), Adams, Gottschling, Kaiser, and Sterns, Cold Spring Harbor Press (1998). Alternatively, antisense technology can be used to reduce the activity of a particular polypeptide. For example, a cell can be engineered to contain a cDNA that encodes an antisense molecule that prevents a polypeptide from being translated. The term
30 "antisense molecule" as used herein encompasses any nucleic acid molecule or nucleic acid analog (e.g., peptide nucleic acids) that contains a sequence that corresponds to the coding strand of an endogenous polypeptide. An antisense molecule also can have

flanking sequences (e.g., regulatory sequences). Thus, antisense molecules can be ribozymes or antisense oligonucleotides. A ribozyme can have any general structure including, without limitation, hairpin, hammerhead, or axhead structures, provided the molecule cleaves RNA. Further, gene silencing can be used to reduce the activity of a particular polypeptide.

A cell having reduced activity of a polypeptide can be identified using any method. For example, enzyme activity assays such as those described herein can be used to identify cells having a reduced enzyme activity.

A polypeptide having (1) the amino acid sequence set forth in SEQ ID NO:39 (the OS17 polypeptide) or (2) an amino acid sequence sharing at least about 60 percent sequence identity with the amino acid sequence set forth in SEQ ID NO:39 can have three functional domains: a domain having CoA-synthetase activity, a domain having 3-HP-CoA dehydratase activity, and a domain having CoA-reductase activity. Such polypeptides can be selectively modified by mutating and/or deleting domains such that one or two of the enzymatic activities are reduced. Reducing the dehydratase activity of the OS17 polypeptide can cause acrylyl-CoA to be created from propionyl-CoA. The acrylyl-CoA then can be contacted with a polypeptide having CoA hydrolase activity to produce acrylate from propionate (Figure 43). Similarly, acrylyl-CoA can be created from 3-HP by using, for example, an OS17 polypeptide having reduced reductase activity.

D. Production of Organic Acids and Related Products via In Vitro Techniques

In addition, purified polypeptides having enzymatic activity can be used alone or in combination with cells to produce 3-HP or other organic compounds such as 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP. For example, a preparation containing a substantially pure polypeptide having 3-hydroxypropionyl-CoA dehydratase activity can be used to catalyze the formation of 3-HP-CoA, a precursor to 3-HP. Further, cell-free extracts containing a polypeptide having enzymatic activity can be used alone or in combination with purified polypeptides and/or cells to produce 3-HP. For example, a cell-free extract containing a

polypeptide having CoA transferase activity can be used to form lactyl-CoA, while a microorganism containing polypeptides have the enzymatic activities necessary to catalyze the reactions needed to form 3-HP from lactyl-CoA can be used to produce 3-HP. Any method can be used to produce a cell-free extract. For example, osmotic shock, sonication, and/or a repeated freeze-thaw cycle followed by filtration and/or centrifugation can be used to produce a cell-free extract from intact cells.

It is noted that a cell, purified polypeptide, and/or cell-free extract can be used to produce 3-HP that is, in turn, treated chemically to produce another compound. For example, a microorganism can be used to produce 3-HP, while a chemical process is used to modify 3-HP into a derivative such as polymerized 3-HP or an ester of 3-HP. Likewise, a chemical process can be used to produce a particular compound that is, in turn, converted into 3-HP or other organic compound (e.g., 1,3-propanediol, acrylic acid, polymerized acrylate, esters of acrylate, esters of 3-HP, and polymerized 3-HP) using a cell, substantially pure polypeptide, and/or cell-free extract described herein. For example, a chemical process can be used to produce acrylyl-CoA, while a microorganism can be used convert acrylyl-CoA into 3-HP.

E. Fermentation of Cells to Produce Organic Acids

Typically, 3-HP is produced by providing a production cell, such as a microorganism, and culturing the microorganism with culture medium such that 3-HP is produced. In general, the culture media and/or culture conditions can be such that the microorganisms grow to an adequate density and produce 3-HP efficiently. For large-scale production processes, any method can be used such as those described elsewhere (Manual of Industrial Microbiology and Biotechnology, 2nd Edition, Editors: A. L. Demain and J. E. Davies, ASM Press; and Principles of Fermentation Technology, P. F. Stanbury and A. Whitaker, Pergamon). Briefly, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing appropriate culture medium with, for example, a glucose carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be

larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose.

Once transferred, the microorganisms can be incubated to allow for the production of 3-HP. Once produced, any method can be used to isolate the 3-HP. For example, common separation techniques can be used to remove the biomass from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange procedures) can be used to obtain the 3-HP from the microorganism-free broth. In addition, 3-HP can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated.

F. Products Created From the Disclosed-Biosynthetic Routes

The organic compounds produced from any of the steps provided in Figures 1-5, 43-44, 54, and 55 can be chemically converted into other organic compounds. For example, 3-HP can be hydrogenated to form 1,3 propanediol, a valuable polyester monomer. Hydrogenating an organic acid such as 3-HP can be performed using any method such as those used to hydrogenate succinic acid and/or lactic acid. For example, 3-HP can be hydrogenated using a metal catalyst. In another example, 3-HP can be dehydrated to form acrylic acid. Any method can be used to perform a dehydration reaction. For example, 3-HP can be heated in the presence of a catalyst (e.g., a metal or mineral acid catalyst) to form acrylic acid. Propanediol also can be created using polypeptides having oxidoreductase activity (e.g., enzymes is the 1.1.1.- class of enzymes) *in vitro* or *in vivo*.

V. Overview of Methodology Used to Create Biosynthetic Pathways That Make 3-HP from PEP

The invention provides methods of making 3-HP and related products from PEP via the use of biosynthetic pathways. Illustrative examples include methods involving the

production of 3-HP via a lactate intermediate, a malonyl-CoA intermediate, and a β -alanine intermediate.

A. Biosynthetic Pathway for Making 3-HP through a Lactic Acid Intermediate

5 A biosynthetic pathway that allows for the production of 3-HP from PEP was constructed (Figure 1). This pathway involved using several polypeptides that were cloned and expressed as described herein. *M. elsdenii* cells (ATCC 17753) were used as a source of genomic DNA. Primers were used to identify and clone a nucleic acid
10 sequence encoding a polypeptide having CoA transferase activity (SEQ ID NO: 1). The polypeptide was subsequently tested for enzymatic activity and found to have CoA transferase activity.

Similarly, PCR primers were used to identify nucleic acid sequences from *M. elsdenii* genomic DNA that encoded an E1 activator, E2 α , and E2 β polypeptides (SEQ
15 ID NOs: 9, 17, and 25, respectively). These polypeptides were subsequently shown to have lactyl-CoA dehydratase activity.

Chloroflexus aurantiacus cells (ATCC 29365) were used as a source of genomic DNA. Initial cloning lead to the identification of nucleic acid sequences: OS17 (SEQ ID
20 NO: 129) and OS19 (SEQ ID NO: 40). Subsequence assays revealed that OS17 encodes a polypeptide having CoA synthase activity, dehydratase activity, and dehydrogenase activity (propionyl-CoA synthetase). Subsequence assays also revealed that OS19 encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity (also referred to as acrylyl-CoA hydratase activity).

Several operons were constructed for use in *E. coli*. These operons allow for the
25 production of 3-HP in bacterial cells. Additional experiments allowed for the expression of these polypeptide in yeast, which can be used to produce 3-HP.

B. Biosynthetic Pathway for Making 3-HP through a Malonyl-CoA Intermediate

30 Another pathway leading to the production of 3-HP from PEP was constructed. This pathway used a polypeptide having acetyl CoA carboxylase activity that was isolated

from *E. coli* (Example 9), and a polypeptide having malonyl-CoA reductase activity that was isolated from *Chloroflexus aurantiacus* (Example 10). The combination of these two polypeptides allows for the production of 3-HP from acetyl-CoA (Figure 44).

- 5 Nucleic acid encoding a polypeptide having malonyl-CoA reductase activity (SEQ ID NO:140) was cloned, sequenced, and expressed. The polypeptide having malonyl-CoA reductase activity was then used to make 3-HP.

C. Biosynthetic Pathways For Making 3-HP through a β -alanine Intermediate

- 10 In general, prokaryotes and eukaryotes metabolize glucose via the Embden-Meyerhof-Parnas pathway to PEP, a central metabolite in carbon metabolism. The PEP generated from glucose is either carboxylated to oxaloacetate or is converted to pyruvate. Carboxylation of PEP to oxaloacetate can be catalyzed by a polypeptide having PEP carboxylase activity, a polypeptide having PEP carboxykinase activity, or a polypeptide
15 having PEP transcarboxylase activity. Pyruvate that is generated from PEP by a polypeptide having pyruvate kinase activity can also be converted to oxaloacetate by a polypeptide having pyruvate carboxylase activity.

- Oxaloacetate generated either from PEP or pyruvate can act as a precursor for production of aspartic acid. This conversion can be carried out by a polypeptide having
20 aspartate aminotransferase activity, which transfers an amino group from glutamate to oxaloacetate. Glutamate consumed in this reaction can be regenerated by the action of a polypeptide having glutamate dehydrogenase activity or by the action of a polypeptide having 4, 4-aminobutyrate aminotransferase activity. The decarboxylation of aspartate to β -alanine is catalyzed by a polypeptide having aspartate decarboxylase activity. β -alanine
25 produced through this biochemistry can be converted to 3-HP via two possible pathways. These pathways are provided in Figures 54 and 55.

- The steps involved in the production of β -alanine can be the same for both pathways. These steps can be accomplished by endogenous polypeptides in the host cells which convert PEP to β -alanine, or these steps can be accomplished with recombinant
30 DNA technology using known polypeptides such as polypeptides having PEP-

carboxykinase activity (4.1.1.32), aspartate aminotransferase activity (2.6.1.1), and aspartate alpha-decarboxylase activity (4.1.1.11).

As depicted in Figure 54, a polypeptide having CoA transferase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:2) can be used to convert β -alanine to β -alanyl-CoA. β -alanyl-CoA can be converted to acrylyl-CoA via a polypeptide having β -alanyl-CoA ammonia lyase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:160). Acrylyl-CoA can be converted to 3-HP-CoA using a polypeptide having 3-HP-CoA dehydratase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:40). 3-HP-CoA can be converted into 3-HP via a polypeptide having CoA transferase activity (e.g., a polypeptide having a sequence set forth in SEQ ID NO:2).

As depicted in Figure 55, a polypeptide having 4,4-aminobutyrate aminotransferase activity (2.6.1.19) can be used to convert β -alanine into malonate semialdehyde. The malonate semialdehyde can be converted into 3-HP using either a polypeptide having 3-hydroxypropionate dehydrogenase activity (1.1.1.59) or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

EXAMPLES

Example 1 – Cloning nucleic acid molecules that encode a polypeptide having CoA transferase activity

Genomic DNA was isolated from *Megasphaera elsdenii* cells (ATCC 17753) grown in 1053 Reinforced Clostridium media under anaerobic conditions at 37°C in roll tubes for 12-14 hours. Once grown, the cells were pelleted, washed with 5 mL of a 10 mM Tris solution, and repelleted. The pellet was resuspended in 1 mL of Gentra Cell Suspension Solution to which 14.2 mg of lysozyme and 4 μ L of 20 mg/mL proteinase K solution was added. The cell suspension was incubated at 37°C for 30 minutes. The genomic DNA was then isolated using a Gentra Genomic DNA Isolation Kit following the provided protocol. The precipitated genomic DNA was spooled and air-dried for 10 minutes. The genomic DNA was suspended in 500 μ L of a 10 mM Tris solution and stored at 4°C.

Two degenerate forward (CoAF1 and CoAF2) and three degenerate reverse (CoAR1, CoAR2, and CoAR3) PCR primers were designed based on conserved acetoacetyl CoA transferase and propionate CoA transferase sequences (CoAF1 5'-GAAWSCGGYSCNATYGGYGG-3', SEQ ID NO: 49; CoAF2 5'-TTYTGYG-GYRSBTTYACBGCWGG-3', SEQ ID NO: 50; CoAR1 5'-CCWGCVGTRAAV-SYRCCRCARAA-3', SEQ ID NO: 51; CoAR2 5'-AARACDSMRCGTTTCVGTRA-TRTA-3', SEQ ID NO: 52; and CoAR3 5'-TCRAYRCCSGGWGCRAYTTC-3', SEQ ID NO: 53). The primers were used in all logical combinations in PCR using Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and 1 ng of genomic DNA per μ L reaction mix. PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 59°C, 4 cycles at 57°C, 4 cycles at 55°C, and 18 cycles at 52°C. Each cycle used an initial 30-second denaturing step at 94°C and a 3 minute extension at 72°C. The program had an initial denaturing step for 2 minutes at 94°C and a final extension step of 4 minutes at 72°C. Time allowed for annealing was 45 seconds.

The amounts of PCR primer used in the reactions were increased 2-8 fold above typical PCR amounts depending on the amount of degeneracy in the 3' end of the primer. In addition, separate PCR reactions containing each individual primer were made to identify PCR products resulting from single degenerate primers. Each PCR product (25 μ L) was separated by electrophoresis using a 1% TAE (Tris-acetate-EDTA) agarose gel.

The CoAF1-CoAR2, CoAF1-CoAR3, CoAF2-CoAR2, and CoAF2-CoAR3 combinations produced a band of 423, 474, 177, and 228 bp, respectively. These bands matched the sizes based on other CoA transferase sequences. No band was visible from the individual primer control reactions. The CoAF1-CoAR3 fragment (474 bp) was isolated and purified using a Qiagen Gel Extraction Kit (Qiagen Inc., Valencia, CA).

Four μ L of the purified band was ligated into pCRII vector and transformed into TOP10 *E. coli* cells by heat-shock using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 μ g/mL of ampicillin (Amp) and 50 μ g/mL of 5-Bromo-4-Chloro-3-Indolyl-*B*-D-Galactopyranoside (X-gal). Single, white colonies were plated onto fresh media and screened in a PCR reaction using the CoAF1 and CoAR3 primers to confirm the presence of the insert.

Plasmid DNA obtained using a QiaPrep Spin Miniprep Kit (Qiagen, Inc) was quantified and used for DNA sequencing with M13R and M13F primers. Sequence analysis revealed that the CoAF1-CoAR3 fragment shared sequence similarity with acetoacetyl CoA transferase sequences.

5 Genome walking was performed to obtain the complete coding sequence. The following primers for genome walking in both upstream and downstream directions were designed using the portion of the 474 bp CoAF1-CoAR3 fragment sequence that was internal to the degenerate primers (COAGSP1F 5'-GAATGTTTACTTCTGCGG-CACCTTCAC-3', SEQ ID NO:54; COAGSP2F 5'-GACCAGATCACTTCAACG-GTTCCTATG-3', SEQ ID NO:55; COAGSP1R 5'-GCATAGGAACCGTTGAAA-GTGATCTGG-3', SEQ ID NO:56; and COAGSP2R 5'-GTTAGTACCGAAGTTG-CTGACGTTGATG-3', SEQ ID NO:57). The COAGSP1F and COAGSP2F primers face downstream, while the COAGSP1R and COAGSP2R primers face upstream. In addition, the COAGSP2F and COAGSP2R primers are nested inside the COAGSP1F and
10 COAGSP1R primers. Genome walking was performed using the Universal Genome Walking kit (ClonTech Laboratories, Inc., Palo Alto, CA) with the exception that additional libraries were generated with enzymes *Nru* I, *Sca* I, and *Hinc* II. First round PCR was conducted in a Perkin Elmer 2400 Thermocycler with 7 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 65°C
15 with a final extension at 65°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. The first and second round product (20 µL) was separated by electrophoresis on a 1% TAE agarose gel. Amplification products were obtained with the *Stu* I library for the reverse direction. The second round
20 product of 1.5 Kb from this library was gel purified, cloned, and sequenced. Sequence analysis revealed that the sequence derived from genome walking overlapped with the CoAF1-CoAR3 fragment and shared sequence similarity with other sequences such as acetoacetyl CoA transferase sequences (Figures 8-9).

25 Nucleic acid encoding the CoA transferase (propionyl-CoA transferase or *pcf*) from *Megasphaera elsdenii* was PCR amplified from chromosomal DNA using following PCR program: 25 cycles of 95°C for 30 seconds to denature, 50°C for 30 seconds to

anneal, and 72°C for 3 minutes for extension (plus 2 seconds per cycle). The primers used were designated PCT-1.114 (5'-ATGAGAAAAGTAGAAATCATTAC-3'; SEQ ID NO:58) and PCT-2.2045 (5'-GGCGGAAGTTGACGATAATG-3'; SEQ ID NO:59). The resulting PCR product (about 2 kb as judged by agarose gel electrophoresis) was purified using a Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified product was ligated to pETBlue-1 using the Perfectly Blunt cloning Kit (Novagen, Madison, WI). The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen, Madison, WI) that were spread on LB agar plates supplemented with 50 µg/mL carbenicillin, 40 µg/mL IPTG, and 40 µg/mL X-Gal. White colonies were isolated and screened for the presence of inserts by restriction mapping. Isolates with the correct restriction pattern were sequenced from each end using the primers pETBlueUP and pETBlueDOWN (Novagen) to confirm the sequence at the ligation points.

The plasmid was transformed into Tuner (DE3) pLacI chemically competent cells (Novagen, Madison, WI), and expression from the construct tested. Briefly, a culture was grown overnight to saturation and diluted 1:20 the following morning in fresh LB medium with the appropriate antibiotics. The culture was grown at 37°C with aeration to an OD₆₀₀ of about 0.6. The culture was induced with IPTG at a final concentration of 100 µM. The culture was incubated for an additional two hours at 37°C with aeration. Aliquots were taken pre-induction and 2 hours post-induction for SDS-PAGE analysis. A band of the expected molecular weight (55,653 Daltons predicted from the sequence) was observed after IPTG treatment. This band was not observed in cells containing a plasmid lacking the nucleic acid encoding the transferase.

Cell free extracts were prepared to assess enzymatic activity. Briefly, the cells were harvested by centrifugation and disrupted by sonication. The sonicated cell suspension was centrifuged to remove cell debris, and the supernatant was used in the assays.

Transferase activity was measured in the following assay. The assay mixture used contained 100 mM potassium phosphate buffer (pH 7.0), 200 mM sodium acetate, 1 mM dithiobisnitrobenzoate (DTNB), 500 µM oxaloacetate, 25 µM CoA-ester substrate, and 3 µg/mL citrate synthase. If present, the CoA transferase transfers the CoA from the CoA ester to acetate to form acetyl-CoA. The added citrate synthase condenses oxaloacetate

and acetyl-CoA to form citrate and free CoASH. The free CoASH complexes with DTNB, and the formation of this complex can be measured by a change in the optical density at 412 nm. The activity of the CoA transferase was measured using the following substrates: lactyl-CoA, propionyl-CoA, acrylyl-CoA, and 3-hydroxypropionyl-CoA. The units/mg of protein was calculated using the following formula:

$$(\Delta E/\text{min} * V_f * \text{dilution factor}) / (V_s * 14.2) = \text{units/mL}$$

where $\Delta E/\text{min}$ is the change in absorbance per minute at 412 nm, V_f is the final volume of the reaction, and V_s is the volume of sample added. The total protein concentration of the cell free extract was about 1 mg/mL so the units/mL equals units/mg.

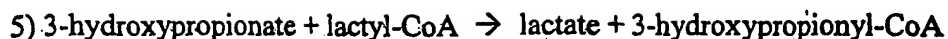
Cell free extracts from cells containing nucleic acid encoding the CoA transferase exhibited CoA transferase activity (Table 2). The observed CoA transferase activity was detected for the lactyl-CoA, propionyl-CoA, acrylyl-CoA, and 3-hydroxypropionyl-CoA substrates (Table 2). The highest CoA transferase activity was detected for lactyl-CoA and propionyl-CoA.

Table 2

Substrate	Units/mg
Lactyl-CoA	211
Propionyl-CoA	144
Acrylyl-CoA	118
3-Hydroxypropionyl-CoA	110

The following assay was performed to test whether the CoA transferase activity can use the same CoA substrate donors as recipients. Specifically, CoA transferase activity was assessed using a Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Voyager RP workstation (PerSeptive Biosystems). The following five reactions were analyzed:

- 1) acetate + lactyl-CoA \rightarrow lactate + acetyl-CoA
- 2) acetate + propionyl-CoA \rightarrow propionate + acetyl-CoA
- 3) lactate + acetyl-CoA \rightarrow acetate + lactyl-CoA
- 4) lactate + acrylyl-CoA \rightarrow acrylate + lactyl-CoA



MALDI-TOF MS was used to measure simultaneously the appearance of the product CoA ester and the disappearance of the donor CoA ester. The assay buffer contained 50 mM potassium phosphate (pH 7.0), 1 mM CoA ester, and 100 mM respective acid salt. Protein from a cell free extract prepared as described above was added to a final concentration of 0.005 mg/mL. A control reaction was prepared from a cell free extract prepared from cells lacking the construct containing the CoA transferase-encoding nucleic acid. For each reaction, the cell free extract was added last to start the reaction. Reactions were allowed to proceed at room temperature and were stopped by adding 1 volume 10% trifluoroacetic acid (TFA). The reaction mixtures were purified prior to MALDI-TOF MS analysis using Sep Pak Vac C₁₈ 50 mg columns (Waters, Inc.). The columns were conditioned with 1 mL methanol and equilibrated with two washes of 1 mL 0.1% TFA. Each sample was applied to the column, and the flow through was discarded. The column was washed twice with 1 mL 0.1% TFA. The sample was eluted in 200 μ L 40% acetonitrile, 0.1% TFA. The acetonitrile was removed by centrifugation *in vacuo*. Samples were prepared for MALDI-TOF MS analysis by mixing 1:1 with 110 mM sinapinic acid in 0.1% TFA, 67% acetonitrile. The samples were allowed to air dry.

In reaction #1, the control sample exhibited a main peak at a molecular weight corresponding to lactyl-CoA (MW 841). There was a minor peak at the molecular weight corresponding to acetyl-CoA (MW 811). This minor peak was determined to be the left-over acetyl-CoA from the synthesis of lactyl-CoA. The reaction #1 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited complete conversion of lactyl-CoA to acetyl-CoA. No peak was observed for lactyl-CoA. This result indicates that the CoA transferase activity can transfer CoA from lactyl-CoA to acetate to form acetyl-CoA.

In reaction #2, the control sample exhibited a dominant peak at a molecular weight corresponding to propionyl-CoA (MW 825). The reaction #2 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a dominant peak at a molecular weight corresponding to acetyl-CoA (MW 811).

No peak was observed for propionyl-CoA. This result indicates that the CoA transferase activity can transfer CoA from propionyl-CoA to acetate to form acetyl-CoA.

In reaction #3, the control sample exhibited a dominant peak at a molecular weight corresponding to acetyl-CoA (MW 811). The reaction #3 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a peak corresponding to lactyl-CoA (MW 841). The peak corresponding to acetyl-CoA did not disappear. In fact, the ratio of the size of the two peaks was about 1:1. The observed appearance of the peak corresponding to lactyl-CoA demonstrates that the CoA transferase activity catalyzes reaction #3.

In reaction #4, the control sample exhibited a dominant peak at a molecular weight corresponding to acrylyl-CoA (MW 823). The reaction #4 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a dominant peak corresponding to lactyl-CoA (MW 841). This result demonstrates that the CoA transferase activity catalyzes reaction #4.

In reaction #5, deuterated lactyl-CoA was used to detect the transfer of CoA from lactate to 3-hydroxypropionate since lactic acid and 3-HP have the same molecular weight as do their respective CoA esters. Using deuterated lactyl-CoA allowed for the differentiation between lactyl-CoA and 3-hydroxypropionate using MALDI-TOF MS. The control sample exhibited a diffuse group of peaks at molecular weights ranging from MW 841 to 845 due to the varying amounts of hydrogen atoms that were replaced with deuterium atoms. In addition, a significant peak was observed at a molecular weight corresponding to acetyl-CoA (MW 811). This peak was determined to be the left-over acetyl-CoA from the synthesis of lactyl-CoA. The reaction #5 sample containing the cell extract from cells transfected with the CoA transferase-encoding plasmid exhibited a dominant peak at a molecular weight corresponding to 3-hydroxypropionyl-CoA (MW 841) as opposed to a group of peaks ranging from MW 841 to 845. This result demonstrates that the CoA transferase catalyzes reaction #5.

**Example 2 – Cloning nucleic acid molecules that encode a
multiple polypeptide complex having lactyl-CoA dehydratase activity**

The following methods were used to clone an E1 activator polypeptide. Briefly, four degenerate forward and five degenerate reverse PCR primers were designed based on conserved sequences of E1 activator protein homologs (E1F1 5'- GCWACBGGY-TAYGGYCG-3', SEQ ID NO:60; E1F2 5'-GTYRTYGAYRTYGGYGGYCAGGA-3', SEQ ID NO:61; E1F3 5'-ATGAACGAYAARTGYGCWGCWGG-3', SEQ ID NO:62; E1F4 5'-TGYGCWGCWGGYACBGGYCGYTT-3', SEQ ID NO:63; E1R1 5'-TCCT-GRCCRCCRAYRTCRAAYRAC-3', SEQ ID NO:64; E1R2 5'-CCWGCWGCRCAY-TTRTCGTTCAT-3', SEQ ID NO:65; E1R3 5'-AARCGRCCVGTGCCWGCWG-CRCA-3', SEQ ID NO:66; E1R4 5'- GCTTCGSWTTTCRACRATGSW-3', SEQ ID NO:67; and E1R5 5'-GSWRATRACCTTCGCWTTTCWGCRAA-3', SEQ ID NO:68).

The primers were used in all logical combinations in PCR using Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and 1 ng of genomic DNA per μ L reaction mix. PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 60°C, 4 cycles at 58°C, 4 cycles at 56°C, and 18 cycles at 54°C. Each cycle used an initial 30-second denaturing step at 94°C and a 3 minute extension step at 72°C. The program had an initial denaturing step for 2 minutes at 94°C and a final extension step of 4 minutes at 72°C. Time allowed for annealing was 45 seconds. The amounts of PCR primer used in the reactions were increased 2-10 fold above typical PCR amounts depending on the amount of degeneracy in the 3' end of the primer. In addition, separate PCR reactions containing each individual primer were made to identify PCR product resulting from single degenerate primers. Each PCR product (25 μ L) was separated by electrophoresis using a 1% TAE (Tris-acetate-EDTA) agarose gel.

The E1F2-E1R4, E1F2-E1R5, E1F3-E1R4, E1F3-E1R5, and E1F4-E1R4R2 combinations produced a band of 195, 207, 144, 156, and 144 bp, respectively. These bands matched the expected size based on E1 activator sequences from other species. No band was visible with individual primer control reactions. The E1F2-E1R5 fragment (207 bp) was isolated and purified using Qiagen Gel Extraction procedure (Qiagen Inc., Valencia, CA). The purified band (4 μ L) was ligated into a *pCRII* vector that then was transformed into TOP10 *E. coli* cells by heat-shock using a TOPO cloning procedure

(Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 $\mu\text{g/mL}$ of ampicillin (Amp) and 50 $\mu\text{g/mL}$ of 5-Bromo-4-Chloro-3-Indolyl-*B*-D-Galactopyranoside (X-gal). Single, white colonies were plated onto fresh media and screened in a PCR reaction using the E1F2 and E1R5 primers to confirm the presence of the insert. Plasmid DNA was obtained from multiple colonies using a QiaPrep Spin Miniprep Kit (Qiagen, Inc). Once obtained, the plasmid DNA was quantified and used for DNA sequencing with M13R and M13F primers. Sequence analysis revealed a nucleic acid sequence encoding a polypeptide and revealed that the E1F2-E1R5 fragment shared sequence similarity with E1 activator sequences (Figures 12-13).

Genome walking was performed to obtain the complete coding sequence of E2 α and β subunits. Briefly, four primers for performing genome walking in both upstream and downstream directions were designed using the portion of the 207 bp E1F2-E1R5 fragment sequence that was internal to the E1F2 and E1R5 degenerate primers (E1GSP1F 5'-ACGTCATGTCGAAGGTACTGGAAATCC-3', SEQ ID NO:69; E1GSP2F 5'-GGGACTGGTACTTCAAATCGAAGCATC-3', SEQ ID NO:70; E1GSP1R 5'-TGACGGCAGCGGGATGCTTCGATTGA-3', SEQ ID NO:71; and E1GSP2R 5'-TCAGACATGGGGATTTCAGTACCTTC-3', SEQ ID NO:72). The E1GSP1F and E1GSP2F primers face downstream, while the E1GSP1R and E1GSP2R primers face upstream. In addition, the E1GSP2F and E1GSP2R primers are nested inside the E1GSP1F and E1GSP1R primers.

Genome walking was performed using the Universal Genome Walking Kit (Clontech Laboratories, Inc., Palo Alto, CA) with the exception that additional libraries were generated with enzymes *Nru* I, *Sca* I, and *Hinc* II. First round PCR was performed in a Perkin Elmer 2400 Thermocycler with 7 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. The first and second round product (20 μL) was separated by electrophoresis using 1% TAE agarose gel. Amplification products were obtained with the *Stu* I library for both forward and reverse directions. The second round product of about 1.5 kb for forward direction and 3 kb fragment for reverse direction from the *Stu* I

library were gel purified, cloned, and sequenced. Sequence analysis revealed that the sequence derived from genome walking overlapped with the E1F2-E1R5 fragment.

To obtain additional sequence, a second genome walk was performed using a first round primer (E1GSPF5 5'-CCGTGTTACTTGGGAAGGTATCGCTGTCTG-3', SEQ ID NO:73) and a second round primer (E1GSPF6 5'-GCCAATGAAGGAGGAAA-CCACTAATGAGTC-3', SEQ ID NO:74). The genome walk was performed using the *Nru*I, *Sca*I, and *Hinc*II libraries. In addition, ClonTech's Advantage-Genomic Polymerase was used for the PCR. First round PCR was performed in a Perkin Elmer 2400 Thermocycler with an initial denaturing step at 94°C for 2 minutes, 7 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 65°C with a final extension at 65°C for 4 minutes. The first and second round product (20 µL) was separated by electrophoresis on a 1% agarose gel. An about 1.5 kb amplification product was obtained from second round PCR of the *Hinc*II library. This band was gel purified, cloned, and sequenced. Sequence analysis revealed that it overlapped with the previously obtained genome walk fragment. In addition, sequence analysis revealed a nucleic acid sequence encoding an E2 α subunit that shares sequence similarities with other sequences (Figures 16-17). Further, sequence analysis revealed a nucleic acid sequence encoding an E2 β subunit that shares sequence similarities with other sequences (Figures 20-21).

Additional PCR and sequence analysis revealed the order of polypeptide encoding sequences within the region containing the lactyl-CoA dehydratase-encoding sequences. Specifically, the E1GSP1F and COAGSP1R primer pair and the COAGSP1F and E1GSP1R primer pair were used to amplify fragments that encode both the CoA transferase and E1 activator polypeptides. Briefly, *M. elsdenii* genome DNA (1 ng) was used as a template. The PCR was conducted in Perkin Elmer 2400 Thermocycler using Long Template Polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The PCR program used was as follows: 94°C for 2 minutes; 29 cycles of 94°C for 30 seconds, 61°C for 45 seconds, and 72°C for 6 minutes; and a final extension of 72°C for 10 minutes. Both PCR products (20 µL) were separated on a 1% agarose gel. An

amplification product (about 1.5 kb) was obtained using the COAGSP1F and E1GSP1R primer pair. This product was gel purified, cloned, and sequenced (Figure 22).

The organization of the *M. elsdenii* operon containing the lactyl-CoA dehydratase-encoding sequences was determined to containing the following polypeptide-encoding sequences in the following order: CoA transferase (Figure 6), ORFX (Figure 23), E1 activator protein of lactyl-CoA dehydratase (Figure 10), E2 α subunit of lactyl-CoA dehydratase (Figure 14), E2 β subunit of lactyl-CoA dehydratase (Figure 18), and truncated CoA dehydrogenase (Figure 25).

The lactyl-CoA dehydratase (lactyl-CoA dehydratase or *lcd*) from *M. elsdenii* was PCR amplified from chromosomal DNA using the following program: 94°C for 2 minutes; 7 cycles of 94°C for 30 seconds, 47°C for 45 seconds, and 72°C for 3 minutes; 25 cycles of 94°C for 30 seconds, 54°C for 45 seconds, and 72°C for 3 minutes; and 72°C for 7 minutes. One primer pair was used (OSNBE1F 5'-GGGAATTCCATATG-AAAACTGTGTATACTCTC-3', SEQ ID NO:75 and OSNBE1R 5'-CGACGGAT-CCTTAGAGGATTTCCGAGAAAGC-3', SEQ ID NO:76). The amplified product (about 3.2 kb) was separated on 1% agarose gel, cut from the gel, and purified with a Qiagen Gel Extraction kit (Qiagen, Valencia, CA). The purified product was digested with *Nde* I and *Bam*HI restriction enzymes and ligated into pET11a vector (Novagen) digested with the same enzymes. The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen) that then were spread on LB agar plates supplemented with 50 μ g/mL carbenicillin. Isolated individual colonies were screened for the presence of inserts by restriction mapping. Isolates with the correct restriction pattern were sequenced from each end using Novagen primers (T7 promoter primer #69348-3 and T7 terminator primer #69337-3) to confirm the sequence at the ligation points.

A plasmid having the correct insert was transformed into Tuner (DE3) pLacI chemically competent cells (Novagen, Madison, WI). Expression from this construct was tested as follows. A culture was grown overnight to saturation and diluted 1:20 the following morning in fresh LB medium with the appropriate antibiotics. The culture was grown at 37°C with aeration to an OD₆₀₀ of about 0.6. The culture was induced with IPTG at a final concentration of 100 μ M. The culture was incubated for an additional two

hours at 37°C with aeration. Aliquots were taken pre-induction and 2 hours post-induction for SDS-PAGE analysis. Bands of the expected molecular weight (27,024 Daltons for the E1 subunit, 48,088 Daltons for the E2 α subunit, and 42,517 Daltons for the E2 β subunit—all predicted from the sequence) were observed. These bands were not
5 observed in cells containing a plasmid lacking the nucleic acid encoding the three components of the lactyl-CoA dehydratase.

Cell free extracts were prepared by growing cells in a sealed serum bottle overnight at 37°C. Following overnight growth, the cultures were induced with 1 mM IPTG (added using anaerobic technique) and incubated an additional 2 hours at 37°C. The
10 cells were harvested by centrifugation and disrupted by sonication under strict anaerobic conditions. The sonicated cell suspension was centrifuged to remove cell debris, and the supernatant was used in the assays. The buffer used for cell resuspension/sonication was 50 mM Tris-HCl (pH 7.5), 200 μ M ATP, 7 mM $Mg(SO_4)$, 4 mM DTT, 1 mM dithionite, and 100 μ M NADH.

15 Dehydratase activity was detected with MALDI-TOF MS. The assay was conducted in the same buffer as above with 1 mM lactyl-CoA or 1 mM acrylyl-CoA added and about 5 mg/mL cell free extract. Prior to MALDI-TOF MS analysis, samples were purified using Sep Pak Vac C_{18} columns (Waters, Inc.) as described in Example 1. The following two reactions were analyzed:

- 20 1) acrylyl-CoA \rightarrow lactyl-CoA
 2) lactyl-CoA \rightarrow acrylyl-CoA

In reaction #1, the control sample exhibited a peak at a molecular weight corresponding to acrylyl-CoA (MW 823). The reaction #1 sample containing the cell
25 extract from cells transfected with the dehydratase-encoding plasmid exhibited a major peak at a molecular weight corresponding to lactyl-CoA (MW 841). This result indicates that the dehydratase activity can convert acrylyl-CoA into lactyl-CoA.

To detect dehydratase activity on lactyl-CoA, reaction #2 was carried out in 80% D_2O . The control sample exhibited a peak at a molecular weight corresponding to lactyl-CoA (MW 841). The reaction #2 sample containing the cell extract from cells transfected
30 with the dehydratase-encoding plasmid revealed a lactyl-CoA peak shifted to a deuterated

form. This result indicates that the dehydratase enzyme is active on lactyl-CoA. In addition, the results from both reactions indicate that the dehydratase enzyme can catalyze the lactyl-CoA \leftrightarrow acrylyl-CoA reaction in both directions.

5 **Example 3 – Cloning nucleic acid molecules that encode**
 a polypeptide having 3-hydroxypropionyl CoA dehydratase activity

Genomic DNA was isolated from *Chloroflexus aurantiacus* cells (ATCC 29365). Briefly, *C. aurantiacus* cells in 920 Chloroflexus medium were grown in 50 mL cultures (Falcon 2070 polypropylene tubes) using an Innova 4230 Incubator, Shaker (New
 10 Brunswick Scientific; Edison, NJ) at 50°C with interior lights. Once grown, the cells were pelleted, washed with 5 mL of a 10 mM Tris solution, and re-pelleted. Genomic DNA was isolated from the pelleted cells using a Gentra Genomic "Puregene" DNA isolation kit (Gentra Systems; Minneapolis, MN). Briefly, the pelleted cells were resuspended in 1 mL Gentra Cell Suspension Solution to which 14.2 mg of lysozyme and
 15 4 μ L of 20 mg/mL proteinase K solution was added. The cell suspension was incubated at 37°C for 30 minutes. The precipitated genomic DNA was recovered by centrifugation at 3500 x g for 25 minutes and air-dried for 10 minutes. The genomic DNA was suspended in 300 μ L of a 10 mM Tris solution and stored at 4°C.

The genomic DNA was used as a template in PCR amplification reactions with
 20 primers designed based on conserved domains of crotonase homologs and a *Chloroflexus aurantiacus* codon usage table. Briefly, two degenerate forward (CRF1 and CRF2) and three degenerate reverse (CRR1, CRR2, and CRR3) PCR primers were designed (CRF1 5'-AAYCGBCCVAARGCNCTSAAYGC-3', SEQ ID NO:77; CRF2: 5'-
 TTYGTBGCNGGYGCNGAYAT-3', SEQ ID NO:78; CRR1 5'-ATRTCNG-
 25 CRCCNGCVACRAA-3', SEQ ID NO:79; CRR2 5'-CCRCCRCCSAGNG-
 CRWARCCRTT-3', SEQ ID NO:80; and CRR3 5'-SSWNGCRATVCGRATRTCAC-
 3', SEQ ID NO:81).

These primers were used in all logical combinations in PCR using Taq polymerase (Roche Molecular Biochemicals; Indianapolis, IN) and 1 ng of the genomic DNA per μ L
 30 reaction mix. The PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 61°C, 4 cycles at 59°C, 4 cycles at 57°C, 4 cycles at 55°C,

and 16 cycles at 52°C. Each cycle used an initial 30-second denaturing step at 94°C and a 3-minute extension step at 72°C. The program also had an initial denaturing step for 2 minutes at 94°C and a final extension step of 4 minutes at 72°C. The time allowed for annealing was 45 seconds. The amounts of PCR primer used in the reaction were increased 4-12 fold above typical PCR amounts depending on the amount of degeneracy in the 3' end of the primer. In addition, separate PCR reactions containing each individual primer were performed to identify amplification products resulting from single degenerate primers. Each PCR product (25 µL) was separated by gel electrophoresis using a 1% TAE (Tris-acetate-EDTA) agarose gel.

10 The CRF1-CRR1 and CRF2-CRR2 combinations produced a unique band of about 120 and about 150 bp, respectively. These bands matched the expected size based on crotonase genes from other species. No 120 bp or 150 bp band was observed from individual primer control reactions. Both fragments (i.e., the 120 bp and 150 bp bands) were isolated and purified using the Qiagen Gel Extraction kit (Qiagen Inc., Valencia, CA). Each purified fragment (4 µL) was ligated into *pCRII* vector that then was transformed into TOP10 *E. coli* cells by a heat-shock method using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 µg/mL of ampicillin (Amp) and 50 µg/mL of 5-Bromo-4-Chloro-3-Indolyl-*B*-D-Galactopyranoside (X-gal). Single, white colonies were plated onto fresh media and screened in a PCR reaction using the CRF1 and CRR1 primers and the CRF2 and CRR2 primers to confirm the presence of the desired insert. Plasmid DNA was obtained from multiple colonies with the desired insert using a QiaPrep Spin Miniprep Kit (Qiagen, Inc.). Once obtained, the DNA was quantified and used for DNA sequencing with M13R and M13F primers. Sequence analysis revealed the presence of two different clones from the PCR product of about 150 bp. Each shared sequence similarity with crotonase and hydratase sequences. The two clones were designated OS17 (157 bp PCR product) and OS19 (151 bp PCR product).

Genome walking was performed to obtain the complete coding sequence of OS17. Briefly, primers for conducting genome walking in both upstream and downstream directions were designed using the portion of the 157 bp CRF2-CRR2 fragment sequence that was internal to the CRF2 and CRR2 degenerate primers (OS17F1 5'-CGCTG-

ATATTCGCCAGTTGCTCGAAG-3', SEQ ID NO:82; OS17F2 5'-CCCATCTTG-
CTTTCCGCAAGATTGAGC-3', SEQ ID NO:83; OS17F3 5'-CAATGGCCCTGCCGA-
ATAACGCCCATCT-3', SEQ ID NO:84; OS17R1 5'-CTTCGAGCAACTGGCGAA-
TATCAGCG-3', SEQ ID NO:85; OS17R2 5'-GCTCAATCTTGCGGAAAGCAAG-
5 ATGGG-3', SEQ ID NO:86; and OS17R3 5'-AGATGGGCGTTATTCGGCAGGGCC-
ATTG-3', SEQ ID NO:87). The OS17F1, OS17F3, and OS17F2 primers face
downstream, while the OS17R2, OS17R3, and OS17R1 primers face upstream.

Genome walking was conducted using the Universal Genome Walking kit
(ClonTech Laboratories, Inc., Palo Alto, CA) with the exception that additional libraries
10 were generated with enzymes *Nru* I, *Fsp* I, and *Hinc* II. The first round PCR was
conducted in a Perkin Elmer 2400 Thermocycler with 7 cycles of 2 seconds at 94°C and 3
minutes at 72°C, and 36 cycles of 2 seconds at 94°C and 3 minutes at 66°C with a final
extension at 66°C for 4 minutes. Second round PCR used 5 cycles of 2 seconds at 94°C
and 3 minutes at 72°C, and 20 cycles of 2 seconds at 94°C and 3 minutes at 66°C with a
15 final extension at 66°C for 4 minutes. The first and second round amplification product
(5 µL) was separated by gel electrophoresis on a 1% TAE agarose gel. After the second
round PCR, an amplification product of about 0.4 kb was obtained with the *Fsp* I library
using the OS17R1 primer in the reverse direction, and an amplification product of about
0.6 kb was obtained with the *Hinc* II library using the OS17F2 primer in the forward
20 direction. These PCR products were cloned and sequenced.

Sequence analysis revealed that the sequences derived from genome walking
overlapped with the CRF2-CRR2 fragment and shared sequence similarity with crotonase
and hydratase sequences.

A second genome walking was performed to obtain additional sequences. Six
25 primers were designed for this second genome walk (OS17F4 5'-AAGCTGGG-
TCTGATCGATGCCATTGCTACC-3', SEQ ID NO:88; OS17F5 5'-CTCGATTATCG-
CCCATCCACGTATCGAG-3', SEQ ID NO:89; OS17F6 5'-TGGATGCAATCCG-
CTATGGCATTATCCACG-3', SEQ ID NO:90; OS17R4 5'-TCATTCAGTGCG-
TTCACCGGCGGATTTGTC-3', SEQ ID NO:91; OS17R5 5'-TCGATCCGGAAGT-
30 AGCGATAGCGTTCGATG-3', SEQ ID NO:92; and OS17R6 5'-CTTGGCTGCAAT-
CTCTTCGAGCACTTCAGG-3', SEQ ID NO:93). The OS17F4, OS17F5, and OS17F6

primers faced downstream, while the OS17R4, OS17R5, and OS17R6 primers faced upstream.

The second genome walk was performed using the same methods described for the first genome walk. After the second round of walking, an amplification product of about 2.3 kb was obtained with a *Hinc II* library using the OS17R5 primer in the reverse direction, and an amplification product of about 0.6 kb was obtained with a *Pvu II* library using the OS17F5 primer in the forward direction. The PCR products were cloned and sequenced. Sequence analysis revealed that the sequences derived from the second genome walking overlapped with the sequence obtained during the first genome walking. In addition, the sequence analysis revealed a sequence with 3572 bp.

A BLAST search revealed that the polypeptide encoded by this sequence shares sequence similarity with polypeptides having three different activities. Specifically, the beginning of the OS17 encoded-polypeptide shares sequence similarity with CoA-synthetases, the middle region of the OS17 encoded-polypeptide shares sequence similarity with enoyl-CoA hydratases, and the end region of the OS17 encoded-polypeptide shares sequence similarity with CoA-reductases.

A third genome walk was performed using four primers (OS17UP-6 5'-CATCAGAGGTAATCACCACCTCGTGCA-3', SEQ ID NO:94; OS17UP-7 5'-AAGTAGTAGGCCACCTCGTCGCCATA-3', SEQ ID NO:95; OS17DN-1 5'-GCCAATCAGGCGCTGATCTATGTTCT-3', SEQ ID NO:96; and OS17DN-2 5'-CTGATCTATGTTCTGGCCTCGGAGGT-3', SEQ ID NO:97). The OS17UP-6 and OS17UP-7 primers face upstream, while the OS17DN-1 and OS17DN-2 primers face downstream. The third genome walk yielded an amplification product of about 1.2 kb with a *Nru I* library using the OS17UP-7 primer in the reverse direction. In addition, amplification products of about 4 kb and about 1.1 kb were obtained with a *Hinc II* and *Fsp I* library, respectively, using the OS17DN-2 primer in the forward direction. Sequence analysis revealed a nucleic acid sequence encoding a polypeptide (Figures 27-28). The complete OS17 gene had 5466 nucleotides and encoded a 1822 amino acid polypeptide. The calculated molecular weight of the OS17 polypeptide from the sequence was 201,346 (pI=5.71).

A BLAST search analysis revealed that the product of the OS17 nucleic acid has three different activities based on sequence similarity to (1) CoA-synthetases at the beginning of the OS17 sequence, (2) 3-HP dehydratases in the middle of the OS17 sequence, and (3) CoA-reductases at the end of the OS17 sequence. Thus, the OS17 clone appeared to encode a single enzyme capable of catalyzing three distinct reactions leading to the direct conversion of 3-hydroxypropionate to propionyl CoA: 3-HP → 3-HP-CoA → acrylyl-CoA → propionyl-CoA.

The OS17 gene from *C. aurantiacus* was PCR amplified from chromosomal DNA using the following conditions: 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds to denature, 54°C for 30 seconds to anneal, and 68°C for 6 minutes for extension; followed by 68°C for 10 minutes for final extension. Two primers were used (OS17F 5'-GGGAATTCCATATGATCGACACTGCG-3', SEQ ID NO:136; and OS17R 5'-CGAAGGATCCAACGATAATCGGCTCAGCAC-3', SEQ ID NO:137). The resulting PCR product (~5.6 Kb) was purified using Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified product was digested with NdeI and BamHI restriction enzymes, heated at 80°C for 20 minutes to inactivate the enzymes; purified using Qiagen PCR purification kit, and ligated into a pET11a vector (Novagen, Madison, WI) previously digested with NdeI and BamHI enzymes. The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen, Madison, WI) that were spread on LB agar plates supplemented with 50 µg/mL carbenicillin. Individual transformants were screened by PCR amplification of the OS17 DNA with the OS17F and OS17R primers and conditions as described above directly from colonies cells. Clones that yielded the 5.6 Kb product were used for plasmid purification with Qiagen QiaPrep Spin Miniprep Kit (Qiagen, Inc). Resulting plasmids were transformed into *E. coli* BL21(DE3) cells, and OS17 polypeptide expression induced. The apparent molecular weight of the OS17 polypeptide according to SDS gel electrophoresis was about 190,000 Da.

To assay OS17 polypeptide function, a 100 mL culture of BL21-DE3/pET11a-OS17 cells was started using 1 mL of overnight grown culture as an inoculum. The culture was grown to an OD of 0.5-0.6 and was induced with 100 µM IPTG. After two and a half hours of induction, the cells were harvested by spinning at 8000 rpm in the

floor-centrifuge. The cells were washed with 10 mM Tris-HCl (pH 7.8) and passed twice through a French Press at a gauge pressure of 1000 psi. The cell debris was removed by centrifugation at 15,000 rpm. The activity of the OS17 polypeptide was measured spectrophotometrically, and the products formed during this enzymatic transformation were detected by LC/MS. The assay mix was as follows (*J. Bacteriol.*, 181:1088-1098 (1999)):

	Reagent	Volume	Final Conc.
	Tris-HCl (1000 mM, 7.8 pH)	10 μ L	50 mM
10	MgCl ₂ (100mM)	10 μ L	5 mM
	ATP (30 mM)	20 μ L	3 mM
	KCl (100 mM)	20 μ L	10 mM
	CoASH (5 mM)	20 μ L	0.5 mM
	NAD(P)H	20 μ L	0.5 mM
15	3-hydroxypropionate	2 μ L	1 mM
	Protein extract (7 mg/mL)	20 (40) μ L	140 μ g
	DI water	78 (58) μ L	
	Total	200 μ L	

20 The initial rate of reaction was measured by monitoring the disappearance of NAD(P)H at 340 nm. The activity of the OS17 polypeptide was measured using 3-HP as the substrate. The units/mL of total protein was calculated using the formula set forth in Example 1. The activity of the expressed OS17 polypeptide was calculated to be 0.061 U/mL of total protein. The reaction products were purified using a Sep Pak Vac column (Waters). The column was conditioned with 1 mL methanol and washed two times with 0.5 mL 0.1% TFA. The sample was then applied to the column, and the column was washed two more times with 0.5 mL 0.1% TFA. The sample was eluted with 200 μ L of 40% acetonitrile, 0.1% TFA. The acetonitrile was removed from the sample by vacuum centrifugation. The reaction products were analyzed by LC/MS.

30 Analyses of thioesters namely propionyl CoA, acrylyl CoA, and 3 HP CoA from the above reaction were carried out using a Waters/Micromass ZQ LC/MS instrument

which had a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) placed in series between the chromatograph and the single quadrupole mass spectrometer. LC separations were made using a 4.6 x 150 mm YMC ods-AQ (3 μ m particles, 120 Å pores) reversed-phase chromatography column at room temperature.

- 5 CoA esters were eluted in Buffer A (25 mM ammonium acetate, 0.5% acetic acid) with a linear gradient of buffer B (acetonitrile, 0.5% acetic acid). A flow rate of 0.25 mL/minute was used, and photodiode array UV absorbance was monitored from 200 to 400 nm. All parameters of the electrospray MS system were optimized and selected based on generation of protonated molecular ions ($[M+H]^+$) of the analytes of interest and
- 10 production of characteristic fragment ions. The following instrumental parameters were used for ESI-MS detection of CoA and organic acid-CoA thioesters in the positive ion mode; Extractor: 1 V; RF lens: 0 V; Source temperature: 100°C; Desolvation temperature: 300°C; Desolvation gas: 500 L/hour; Cone gas: 40 L/hour; Low mass resolution: 13.0; High mass resolution: 14.5; Ion energy: 0.5; Multiplier: 650.
- 15 Uncertainties for mass charge ratios (m/z) and molecular masses are $\pm 0.01\%$.

The enzyme assay mix from strains expressing the OS17 polypeptide exhibited peaks for propionyl CoA, acrylyl CoA, and 3-HP CoA with the propionyl CoA peak being the dominant peak. These peaks were missing in the enzyme assay mix obtained from the control strain, which carried vector pET11a without an insert. These results

20 indicate that the OS17 polypeptide has CoA synthetase activity, CoA hydratase activity, and dehydrogenase activity.

Genome walking also was performed to obtain the complete coding sequence of OS19. Briefly, primers for conducting genome walking in both upstream and downstream directions were designed using the portion of the 151 bp CRF2-CRR2

25 fragment sequence that was internal to the CRF2 and CRR2 degenerate primers (OS19F1 5'-GGCTGATATCAAAGCGATGGCCAATGC-3', SEQ ID NO:98; OS19F2 5'-CCAC-GCCTATTGATATGCTCACCAGTG-3', SEQ ID NO:99; OS19F3 5'-GCAAACCGG-TGATTGCTGCCGTGAATGG-3', SEQ ID NO:100; OS19R1 5'-GCATTGGCCAT-CGCTTTGATATCAGCC-3', SEQ ID NO:101; OS19R2 5'-CACTGGTGAGCATATC-

30 AATAGGCGTGG-3', SEQ ID NO:102; and OS19R3 5'-CCATTACGGCAGCAA-

TCACCGGTTTGC-3', SEQ ID NO:103). The OS19F1, OS19F2, and OS19F3 primers face downstream, while the OS19R1, OS19R2, and OS19R3 primers face upstream.

An amplification product of about 0.25 kb was obtained with the *Fsp* I library using the OS19R1 primer, while an amplification product of about 0.65 kb was obtained with the *Pvu* II library using the OS19R1 primer. In addition, an amplification product of about 0.4 kb was obtained with the *Pvu* II library using the OS19F3 primer. The PCR products were cloned and sequenced. Sequence analysis revealed that the sequences derived from genome walking overlapped with the CRF2-CRR2 fragment and shared sequence similarity with crotonase and hydratase sequences. The obtained sequences accounted for most of the coding sequence including the start codon.

A second genome walk was performed to obtain additional sequence using two primers (OS19F7 5'-TCATCATCGCCAGTGAAAACGCGCAGTTCG-3', SEQ ID NO:104 and OS19F8 5'-GGATCGCGCAAACCATTGCCACCAAATCAC-3', SEQ ID NO:105). The OS19F7 and OS19F8 primers face downstream.

An amplification product (about 0.7 kb) obtained from the *Pvu* II library was cloned and sequenced. Sequence analysis revealed that the sequence derived from the second genome walk overlapped with the sequence obtained from the first genome walk and contained the stop codon. The full-length OS19 clone was found to share sequence similarity with other sequences such as crotonase and enoyl-CoA hydratase sequences (Figures 32-33).

The OS19 clone was found to encode a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity also referred to as acrylyl-CoA hydratase activity. The nucleic acid encoding the OS19 dehydratase from *C. aurantiacus* was PCR amplified from chromosomal DNA using the following conditions: 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds to denature, 56°C for 30 seconds to anneal, and 68°C for 1 minute for extension; and 68°C for 5 minutes for final extension. Two primers were used (OSACH3 5'-ATGAGTGAAGAGTCTCTGGTTCTCAGC-3', SEQ ID NO:106 and OSACH2 5'-AGATCGCAATCGCTCGTGTATGTC-3', SEQ ID NO:107).

The resulting PCR product (about 1.2 kb) was separated by agarose gel electrophoresis and purified using Qiagen PCR purification kit (Qiagen Inc.; Valencia, CA). The purified product was ligated into pETBlue-1 using the Perfectly Blunt cloning

Kit (Novagen; Madison, WI). The ligation reaction was transformed into NovaBlue chemically competent cells (Novagen, Madison, WI) that then were spread on LB agar plates supplemented with 50 µg/mL carbenicillin, 40 µg/mL IPTG, and 40 µg/mL X-Gal. White colonies were isolated and screened for the presence of inserts by restriction mapping. Isolates with the correct restriction pattern were sequenced from each end using the primer pETBlueUP and pETBlueDOWN (Novagen) to confirm the sequence at the ligation points.

The plasmid containing the OS19 dehydratase-encoding sequence was transformed into Tuner (DE3) pLacI chemically competent cells (Novagen, Madison, WI), and expression from the construct tested. Briefly, a culture was grown overnight to saturation and diluted 1:20 the following morning in fresh LB medium with the appropriate antibiotics. The culture was grown at 37°C and 250 rpm to an OD₆₀₀ of about 0.6. At this point, the culture was induced with IPTG at a final concentration of 1 mM. The culture was incubated for an additional two hours at 37°C and 250 rpm. Aliquots were taken pre-induction and 2 hours post-induction for SDS-PAGE analysis. A band of the expected molecular weight (27,336 Daltons predicted from the sequence) was observed. This band was not observed in cells containing a plasmid lacking the nucleic acid encoding the hydratase.

~~Cell free extracts were prepared by growing cells as described above. The cells~~ were harvested by centrifugation and disrupted by sonication. The sonicated cell suspension was centrifuged to remove cell debris, and the supernatant was used in the assays. The ability of the 3-hydroxypropionyl-CoA dehydratase to perform the following three reactions was measured using MALDI-TOF MS:

- 1) acrylyl-CoA → 3-hydroxypropionyl-CoA
- 2) 3-hydroxypropionyl-CoA → acrylyl-CoA
- 3) crotonyl-CoA → 3-hydroxybutyryl-CoA

The assay mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM CoA ester, and about 1 µg cell free extract. Reactions were allowed to proceed at room temperature and were stopped by adding 1 volume 10% trifluoroacetic acid (TFA). The reaction mixtures were purified prior to MALDI-TOF MS analysis using Sep Pak Vac C₁₈ 50 mg columns

(Waters, Inc.). The columns were conditioned with 1 mL methanol and then equilibrated with two washes of 1 mL 0.1% TFA. The sample was applied to the column, and the flow through was discarded. The column was washed twice with 1 mL 0.1% TFA. The sample was eluted in 200 μ L 40% acetonitrile, 0.1% TFA. The acetonitrile was removed by centrifugation *in vacuo*. Samples were prepared for MALDI-TOF MS analysis by mixing 1:1 with 110 mM sinapinic acid in 0.1% TFA, 67% acetonitrile. The samples were allowed to air dry.

The conversion of acrylyl-CoA into 3-hydroxypropionyl-CoA catalyzed by the 3-hydroxypropionyl-CoA dehydratase was detected using the MALDI-TOF MS technique. In reaction #1, the control sample exhibited a dominant peak at a molecular weight corresponding to acrylyl-CoA (MW 823). The reaction #1 sample containing the cell extract from cells transfected with the 3-hydroxypropionyl-CoA dehydratase-encoding plasmid exhibited a dominant peak corresponding to 3-hydroxypropionyl-CoA (MW 841). This result demonstrates that the 3-hydroxypropionyl-CoA dehydratase activity catalyzes reaction #1.

To detect the conversion of 3-hydroxypropionyl-CoA into acrylyl-CoA, reaction #2 was carried out in 80% D₂O. The reaction #2 sample containing the cell extract from cells transfected with the 3-hydroxypropionyl-CoA dehydratase-encoding plasmid revealed incorporation of deuterium in the 3-hydroxypropionyl-CoA molecule. This result indicates that the 3-hydroxypropionyl-CoA dehydratase enzyme catalyzes reaction #2. In addition, the results from both #1 and #2 reactions indicate that the 3-hydroxypropionyl-CoA dehydratase enzyme can catalyze the 3-hydroxypropionyl-CoA \leftrightarrow acrylyl-CoA reaction in both directions. It is noted that for both the #1 and #2 reactions, a peak was observed at MW 811, due to leftover acetyl-CoA from the synthesis of 3-hydroxypropionyl-CoA from 3-hydroxypropionate and acetyl-CoA.

The assays assessing conversion of crotonyl-CoA into 3-hydroxybutyryl-CoA also were carried out in 80% D₂O. In reaction #3, the control sample exhibited a dominant peak at a molecular weight corresponding to crotonyl-CoA (MW 837). This result indicated that the crotonyl-CoA was not converted into other products. The reaction #3 sample containing the cell extract from cells transfected with the 3-hydroxypropionyl-CoA dehydratase-encoding plasmid exhibited a diffuse group of peaks corresponding to

deuterated 3-hydroxybutyryl-CoA (MW 855 to MW 857). This result demonstrates that the 3-hydroxypropionyl-CoA dehydratase activity catalyzes reaction #3.

A series of control reactions were performed to confirm the specificity of the 3-hydroxypropionyl-CoA dehydratase. Lactyl-CoA (1 mM) was added to the reaction mixture containing 100 mM Tris (pH 7.0) both in the presence and the absence of the 3-hydroxypropionyl-CoA dehydratase. In both cases, the dominant peak observed had a molecular weight corresponding to lactyl-CoA (MW 841). This result indicates that lactyl-CoA is not affected by the presence of 3-hydroxypropionyl-CoA dehydratase activity even in the presence of D₂O meaning that the 3-hydroxypropionyl-CoA dehydratase enzyme does not attach a hydroxyl group at the alpha carbon position. The presence of 3-hydroxypropionyl-CoA in an 80% D₂O reaction mixture resulted in a shift upon addition of the 3-hydroxypropionyl-CoA dehydratase activity. In the absence of 3-hydroxypropionyl-CoA dehydratase activity, a peak corresponding to 3-hydroxypropionyl-CoA was observed in addition to a peak of MW 811. The MW 811 peak was due to leftover acetyl-CoA from the synthesis of 3-hydroxypropionyl-CoA. In the presence of 3-hydroxypropionyl-CoA dehydratase activity, a peak corresponding to deuterated 3-hydroxypropionyl-CoA was observed (MW 842) due to exchange of a hydroxyl group during the conversion of 3-hydroxypropionyl-CoA to acrylyl-CoA and visa-versa. These control reactions demonstrate that the 3-hydroxypropionyl-CoA dehydratase enzyme is active on 3-hydroxypropionyl-CoA and not active on lactyl-CoA. In addition, these results demonstrate that the product of the acrylyl-CoA reaction is 3-hydroxypropionyl-CoA not lactyl-CoA.

Example 4 - Construction of operon #1

The following operon was constructed and can be used to produce 3-HP in *E. coli* (Figure 34). Briefly, the operon was cloned into a pET-11a expression vector under the control of a T7 promoter (Novagen, Madison, WI). The pET-11a expression vector is a 5677 bp plasmid that uses the ATG sequence of an *NdeI* restriction site as a start codon for inserted downstream sequences.

Nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were

used to amplify the CoA transferase-encoding sequence (OSNBpctF 5'-GGGAATTCC-ATATGAGAAAAGTAGAAATCATTACAGCTG-3', SEQ ID NO:108 and OSCTE-2 5'-GAGAGTATACACAGTTTTACCTCCTTTACAGCAGAGAT-3', SEQ ID NO:109), and two primers were used to amplify the lactyl-CoA dehydratase-encoding
5 sequence (OSCTE-1 5'-ATCTCTGCTGTAAAGGAGGTGAAAAGTGTGTATACTCTC-3', SEQ ID NO:110 and OSEBH-2 5'-ACGTTGATCTCCTTGTACATTAGAGGATTTCCGAGAAAGC-3', SEQ ID NO:111). A nucleic acid molecule encoding a 3-hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSEBH-1 5'-GCTTTCTCGG-
10 AAATCCTCTAATGTACAAGGAGATCAACGT-3', SEQ ID NO:112 and OSHBR 5'-CGACGGATCCTCAACGACCACTGAAGTTGG-3', SEQ ID NO:113).

PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix
15 ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.;
Valencia, CA).

20 The CoA transferase, lactyl-CoA dehydratase (E1, E2 α subunit, and E2 β subunit), and 3-hydroxypropionyl-CoA dehydratase PCR products were assembled using PCR. The OSCTE-1 and OSCTE-2 primers as well as the OSEBH-1 and OSEBH-2 primers were complementary to each other. Thus, the complementary DNA ends could
25 anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSHBR) were added to the assembly PCR mixture, which contained 100 ng of each PCR product (i.e., the PCR products from the CoA-transferase, lactyl-CoA dehydratase, and 3-hydroxypropionyl-CoA dehydratase reactions) as well as the rTth polymerase/Pfu Turbo
30 polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 6 minutes; and a final extension at 68°C for 7 minutes. The assembled PCR

product was gel purified and digested with restriction enzymes (*NdeI* and *BamHI*). The sites for these restriction enzymes were introduced into the assembled PCR product using the OSNBpctF (*NdeI*) and OSHBR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactive the restriction enzymes and used directly for
 5 ligation into pET-11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase
 10 (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once heat shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and analyzed by
 15 digestion with *NdeI* and *BamHI* restriction enzymes.

Example 5 - Construction of operon #2

The following operon was constructed and can be used to produce 3-HP in *E. coli* (Figure 35A and B). Nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR.
 20 Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSCTE-2), and two primers were used to amplify the lactyl-CoA dehydratase-encoding sequence (OSCTE-1 and OSNBe1R 5'-CGACGGATCCTTAGAGGATT-CCGAGAAAGC-3', SEQ ID NO:114). A nucleic acid molecule encoding a 3-
 25 hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSXNhF 5'-GGTGTCT-AGAGACAGTCCTGTCGTTTATGTAGAAGGAG-3', SEQ ID NO:115 and OSXNhR 5'-GGGAATTCCATATGCGTAACTTCCTCCTGCTATCAACGACCACTGAA-GTTGG-3', SEQ ID NO:116).

30 PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and

Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The
5 obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The CoA transferase and lactyl-CoA dehydratase (E1, E2 α subunit, and E2 β subunit) PCR products were assembled using PCR. The OSCTE-1 and OSCTE-2 primers were complementary to each other. Thus, the 22 nucleotides at the end of the CoA
10 transferase sequence and the 22 nucleotides at the beginning of the lactyl-CoA dehydratase could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSNBelR) were added to the assembly PCR mixture, which contained 100 ng of the CoA transferase PCR product, 100 ng of lactyl-CoA dehydratase PCR product, and the
15 rTth polymerase/Pfu Turbo polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 5 minutes; and a final extension at 68°C for 6 minutes.

The assembled PCR product was gel purified and digested with restriction
20 enzymes (*NdeI* and *BamHI*). The sites for these restriction enzymes were introduced into the assembled PCR product using the OSNBpctF (*NdeI*) and OSNBelR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET-11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel
25 purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a
30 heat-shock method. Once heat shocked, the cells were plated on LB plates supplemented with 50 μ g/mL carbenicillin. The plasmid DNA was purified from individual colonies

using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and analyzed by digestion with *NdeI* and *BamHI* restriction enzymes. The digest revealed that the DNA fragment containing CoA transferase-encoding and lactyl-CoA dehydratase-encoding sequences was cloned into the pET-11a vector.

5 The plasmid carrying the CoA transferase-encoding and lactyl-CoA dehydratase-encoding sequences (pTD) was digested with *XbaI* and *NdeI* restriction enzymes, gel purified, and used for cloning the 3-hydroxypropionyl-CoA dehydratase-encoding product upstream of the CoA transferase-encoding sequence. Since this *XbaI* and *NdeI* digest eliminated a ribosome-binding site (RBS) from the pET-11a vector, a new
10 homologous RBS was cloned into the plasmid together with the 3-hydroxypropionyl-CoA dehydratase-encoding product. Briefly, the 3-hydroxypropionyl-CoA dehydratase-encoding PCR-product was digested with *XbaI* and *NdeI* restriction enzymes, heated at 65°C for 30 minutes to inactivate the restriction enzymes, and ligated into pTD. The
15 ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 µg/mL carbenicillin.

Individual colonies were selected, and the plasmid DNA obtained using a Qiagen Spin Miniprep Kit. The obtained plasmids were digested with *XbaI* and *NdeI* restriction enzymes and analyzed by gel electrophoresis. pTD plasmids containing the inserted 3-
20 hydroxypropionyl-CoA dehydratase-encoding PCR product were named pHTD. While expression of the lactyl-CoA hydratase, CoA transferase, and 3-hydroxypropionyl-CoA dehydratase sequences from pHTD was directed by a single T7 promoter, each coding
25 sequence had an individual RBS upstream of their start codon.

To ensure the correct assembly and cloning of the lactyl-CoA hydratase, CoA transferase, and 3-hydroxypropionyl-CoA dehydratase sequences into one operon, both
25 ends of the operon and all junctions between the coding sequences were sequenced. This DNA analysis revealed that the operon was assembled correctly.

The pHTD plasmid was transformed into BL21(DE3) cells to study the expression of the encoded sequences.

Example 6 - Construction of operons #3 and #4

Operon #3 (Figure 36A and B) and operon #4 (Figure 37A and B) each position the E1 activator at the end of the operon. Operon #3 contains a RBS between the 3-hydroxypropionyl-CoA dehydratase-encoding sequence and the E1 activator-encoding sequence. In operon #4, however, the stop codon of the 3-hydroxypropionyl-CoA dehydratase-encoding sequence is fused with the start codon of the E1 activator-encoding sequence as follows: TAGTG. The absence of the RBS in operon #4 can decrease the level of E1 activator expression.

To construct operon #3, nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSHTR 5'-ACGTTGATCTCCTTCTACATTATTTTTTCAGT-CCCATG-3', SEQ ID NO:117), two primers were used to amplify the E2 α and β subunits of the lactyl-CoA dehydratase-encoding sequence (OSEIIXNF 5'-GGTGTCTAGAGTCAAAGGAGAGAACAAAATCATGAGTG-3', SEQ ID NO:118 and OSEIIXNR 5'-GGGAATTCCATATGCGTAACTTCCTCCTGCTATTAGAGGATTTCCGAGAAAGC-3', SEQ ID NO:119), and two primers were used to amplify the E1 activator of the lactyl-CoA dehydratase-encoding sequence (OSHrEIF 5'-TCAGTG-GTCGTTGATCACGCTATAAAGAAAGGTGAAAACGTGTGTATACTCTC-3', SEQ ID NO:120 and OSEIBR 5'-CGACGGATCCCTTCCTTGGAGCTCATGCTTTC-3', SEQ ID NO:121). A nucleic acid molecule encoding a 3-hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSTHF 5'-CATGGGACTGAAAAAATAATGTAGAAGGAGATCAACGT-3', SEQ ID NO:122 and OSEIhHR 5'-GAGAGTATACACAGTTTTCA-CCTTTCTTTATAGCGTGATCAACGACCACTGA-3', SEQ ID NO:123).

PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The

obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The 3-hydroxypropionyl-CoA dehydratase and E1 activator PCR products were assembled using PCR. The OSHrE1F and OSE1rHR primers were complementary to each other. Thus, the primers could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSTHF and OSE1BR) were added to the assembly PCR mixture, which contained 100 ng of the 3-hydroxypropionyl-CoA dehydratase PCR product, 100 ng of E1 activator PCR product, and the rTth polymerase/Pfu Turbo polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 1.5 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and used in a second assembly PCR with gel purified the CoA transferase PCR product. The OSTHF and OSHTR primers were complementary to each other. Thus, the complementary DNA ends could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSEIBR) were added to the second assembly PCR mixture, which contained 100 ng of the purified 3-hydroxypropionyl-CoA dehydratase/E1 PCR assembly, 100 ng of the purified CoA transferase PCR product, and the polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 3 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and digested with *NdeI* and *BamHI* restriction enzymes. The sites for these restriction enzymes were introduced into the assembled PCR products with the OSNBpctF (*NdeI*) and OSEIBR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the

assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once heat shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA). The resulting plasmids carrying the CoA transferase, 3-hydroxypropionyl-CoA dehydratase, and EI activator sequences (pTHrEI) were digested with *XbaI* and *NdeI*, purified using gel electrophoresis and a Qiagen Gel Extraction kit, and used as a vector for cloning of the E2 α subunit/E2 β subunit PCR product.

The E2 α subunit/E2 β subunit PCR product was digested with the same enzymes and ligated into the pTHrEI vector. The ligation reaction was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and digested with *XbaI* and *NdeI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the constructed operon #3 (pEIITHrEI) were transformed into BL21(DE3) cells to study the expression of the cloned sequences. Electrospray mass spectrometry assay confirmed that extracts from these cells have CoA transferase activity and 3-hydroxypropionyl-CoA dehydratase activity. Similar assays are used to confirm that extracts from these cells also have lactyl-CoA dehydratase activity.

To construct operon #4, nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSHTR), two primers were used to amplify the E2 α and β subunits of the lactyl-CoA dehydratase-encoding sequence (OSEIXNF and OSEIXNR), and two primers were used to amplify the EI activator of the lactyl-CoA dehydratase-encoding sequence (OSHEIF 5'-CCAACTTCAGTGGTCGTTAGTGAAAAGTGTGTAT-
ACTCTC-3', SEQ ID NO:124 and OSEIBR). A nucleic acid molecule encoding a 3-

hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSTHF and OSEIHR 5'-GAGAGTATACACAGTTTTTCTACTAACGACCACTGAAGTTGG-3', SEQ ID NO:125).

5 PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C
10 for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The 3-hydroxypropionyl-CoA dehydratase and E1 activator PCR products were assembled using PCR. The OSHEIF and OSEIHR primers were complementary to each
15 other. Thus, the primers could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSTHF and OSE1BR) were added to the assembly PCR mixture, which contained 100 ng of the 3-hydroxypropionyl-CoA dehydratase PCR product, 100 ng of E1 activator PCR product, and the rTth polymerase/Pfu Turbo polymerase mix described above. The
20 following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 1.5 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and used in a second assembly PCR with gel purified the CoA transferase PCR product. The OSTHF and OSHTR primers
25 were complementary to each other. Thus, the complementary DNA ends could anneal to each other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSEIBR) were added to the second assembly PCR mixture, which contained 100 ng of the purified 3-hydroxypropionyl-CoA dehydratase/EI PCR assembly, 100 ng of the purified CoA
30 transferase PCR product, and the polymerase mix described above. The following PCR conditions were used to assemble the products: 94°C for 1 minute; 20 cycles of 94°C for

30 seconds, 54°C for 30 seconds, and 68°C for 3 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and digested with *NdeI* and *BamHI* restriction enzymes. The sites for these restriction enzymes were introduced into the assembled PCR products with the OSNBpctF (*NdeI*) and OSEIBR (*BamHI*) primers. The digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). The resulting plasmids carrying the CoA transferase, 3-hydroxypropionyl-CoA dehydratase, and EI activator sequences (pTHE1) were digested with *XbaI* and *NdeI*, purified using gel electrophoresis and a Qiagen Gel Extraction kit, and used as a vector for cloning of the E2 α subunit/E2 β subunit PCR product.

The E2 α subunit/E2 β subunit PCR product was digested with the same enzymes and ligated into the pTHE1 vector. The ligation reaction was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA) and digested with *XbaI* and *NdeI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the constructed operon #4 (pEIITHEI) were transformed into BL21(DE3) cells to study the expression of the cloned sequences. Electrospray mass spectrometry assays confirmed that extracts from these cells have CoA transferase activity and 3-hydroxypropionyl-CoA dehydratase activity.

Similar assays are used to confirm that extracts from these cells also have lactyl-CoA dehydratase activity.

E. coli plasmid pEIITHrEI carrying a synthetic 3-HP operon was digested with *Nru*I, *Xba*I and *Bam*HI restriction enzymes, *Xba*I-*Bam*HI DNA fragment was gel purified with Quagen Gel Extraction Kit (Qiagen, Inc., Valencia CA) and used for further cloning into Bacillus vector pWH1520 (MoBiTec BmbH, Gottingen, Germany). Vector pWH1520 was digested with *Spe*I and *Bam*HI restriction enzymes and gel purified with Qiagen Gel Extraction Kit. The *Xba*I-*Bam*HI fragment carrying 3-HP operon was ligated into WH1520 vector at 16°C overnight using T4 ligase. The ligation mixture was transformed into chemically competent TOP 10 cells and plated on LB plates supplemented with 50 µg/ml carbenicillin. One clone named *B. megaterium* (pBPO26) was used for assays of CoA-transferase and CoA-hydratase activities. The assays were performed as described above for *E. Coli*. The enzymatic activity was 5 U/mg and 13 U/mg respectively.

Example 7 - Construction of a two plasmid system

The following constructs were constructed and can be used to produce 3-HP in *E. coli* (Figure 38A and B). Nucleic acid molecules encoding a CoA transferase and a lactyl-CoA dehydratase were amplified from *Megasphaera elsdenii* genomic DNA by PCR. Two primers were used to amplify the CoA transferase-encoding sequence (OSNBpctF and OSHTR), two primers were used to amplify the E2 α and β subunits of the lactyl-CoA dehydratase-encoding sequence (OSEIIXNF and OSEIIXNR), and two primers were used to amplify the E1 activator of the lactyl-CoA dehydratase-encoding sequence (E1PROF 5'-GTCGCAGAATTCCCATCAATCGCAGCAATCCCAAC-3', SEQ ID NO:126 and E1PROR 5'-TAACATGGTACCGACAGAAGCGGACCAGCA-AACGA-3', SEQ ID NO:127). A nucleic acid molecule encoding a 3-hydroxypropionyl-CoA dehydratase was amplified from *Chloroflexus aurantiacus* genomic DNA of by PCR using two primers (OSTHF and OSHBR 5'-CGACGGATCCTCAACGACCA-CTGAAGTTGG-3', SEQ ID NO:128).

PCR was conducted in a Perkin Elmer 2400 Thermocycler using 100 ng of genomic DNA and a mix of rTth polymerase (Applied Biosystems; Foster City, CA) and

Pfu Turbo polymerase (Stratagene; La Jolla, CA) in 8:1 ratio. The polymerase mix ensured higher fidelity of the PCR reaction. The following PCR conditions were used: initial denaturation step of 94°C for 2 minutes; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2 minutes; and a final extension at 68°C for 5 minutes. The
5 obtained PCR products were gel purified using a Qiagen Gel Extraction Kit (Qiagen, Inc.; Valencia, CA).

The CoA transferase PCR product and the 3-hydroxypropionyl-CoA dehydratase PCR product were assembled using PCR. The OSTHF and OSHTR primers were complementary to each other. Thus, the complementary DNA ends could anneal to each
10 other during the PCR reaction extending the DNA in both direction. To ensure the efficiency of the assembly, two end primers (OSNBpctF and OSHBR) were added to the assembly PCR mixture, which contained 100 ng of the purified CoA transferase PCR product, 100 ng of the purified 3-hydroxypropionyl-CoA dehydratase PCR product, and the polymerase mix described above. The following PCR conditions were used to
15 assemble the products: 94°C for 1 minute; 20 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 68°C for 2.5 minutes; and a final extension at 68°C for 5 minutes.

The assembled PCR product was gel purified and digested with *NdeI* and *BamHI* restriction enzymes. The sites for these restriction enzymes were introduced into the assembled PCR products with the OSNBpctF (*NdeI*) and OSHBR (*BamHI*) primers. The
20 digested PCR product was heated at 80°C for 30 minutes to inactivate the restriction enzymes and used directly for ligation into a pET11a vector.

The pET-11a vector was digested with *NdeI* and *BamHI* restriction enzymes, gel purified using a Qiagen Gel Extraction kit, treated with shrimp alkaline phosphatase (Roche Molecular Biochemicals; Indianapolis, IN) and used in a ligation reaction with the
25 assembled PCR product. The ligation was performed at 16°C overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into NovaBlue chemically competent cells (Novagen; Madison, WI) using a heat-shock method. Once shocked, the cells were plated on LB plates supplemented with 50 µg/mL carbenicillin. The plasmid DNA was purified from individual colonies using a
30 QiaPrep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA) and digested with *NdeI* and *BamHI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids

carrying the CoA transferase and 3-hydroxypropionyl-CoA dehydratase (pTH) were digested with *XbaI* and *NdeI*, purified using gel electrophoresis and a Qiagen Gel Extraction kit, and used as a vector for cloning of the E2 α subunit/E2 β subunit PCR product.

5 The E2 α subunit/E2 β subunit PCR product digested with the same enzymes was ligated into the pTH vector. The ligation reaction was performed at 16°C overnight using T4 ligase (Roche-Molecular Biochemicals; Indianapolis, IN). The ligation mixture was transformed into chemically competent NovaBlue cells (Novagen) that then were plated on LB plates supplemented with 50 μ g/mL carbenicillin. The plasmid DNA was purified
10 from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA) and digested with *XbaI* and *NdeI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the E2 α and β subunits of the lactyl-CoA dehydratase, the CoA transferase, and the 3-hydroxypropionyl-CoA dehydratase (pEIITH) were
~~transformed into BL21(DE3) cells to study the expression of the cloned sequences.~~

15 The gel purified E1 activator PCR product was digested with *EcoRI* and *KpnI* restriction enzymes, heated at 65°C for 30 minutes, and ligated into a vector (pPROLar.A) that was digested with *EcoRI* and *KpnI* restriction enzymes, gel purified using Qiagen Gel Extraction kit, and treated with shrimp alkaline phosphatase (Roche
~~Molecular Biochemicals; Indianapolis, IN).~~ The ligation was performed at 16°C
20 overnight using T4 ligase (Roche Molecular Biochemicals; Indianapolis, IN). The resulting ligation reaction was transformed into DH10B electro-competent cells (Gibco Life Technologies; Gaithersburg, MD) using electroporation. Once electroporated, the cells were plated on LB plates supplemented with 25 μ g/mL kanamycin. The plasmid DNA was purified from individual colonies using a QiaPrep Spin Miniprep Kit (Qiagen
25 Inc., Valencia, CA) and digested with *EcoRI* and *KpnI* restriction enzymes for gel electrophoresis analysis. The resulting plasmids carrying the E1 activator (pPROEI) are transformed into BL21(DE3) cells to study the expression of the cloned sequence.

 The pPROEI and pEIITH plasmids are compatible plasmids that can be used in the same bacterial host cell. In addition, the expression from the pPROEI and pEIITH
30 plasmids can be induced at different levels using IPTG and arabinose, allowing for the fine-tuning of the expression of the cloned sequences.

Example 8 - Production of 3-HP

3-HP was produced using recombinant *E. coli* in a small-scale batch fermentation reaction. The construction of strain ALS848 (also designated as TA3476 (*J. Bacteriol.*, 143:1081-1085(1980))) that carried inducible T7 RNA polymerase was performed using
5 λ DE3 lysogenization kit (Novagen, Madison, WI) according to the manufacture's instructions. The constructed strain was designated ALS484(DE3). Strain ALS484(DE3) was transformed with pEIITHrEI plasmid using standard electroporation techniques. The transformants were selected on LB/carbenicillin (50 μ g/mL) plates. A single colony was used to inoculate 4 mL culture in a 15 mL culture tube. Strain ALS484(DE3) strain
10 carrying vector pET11a was used as a control. The cells were grown at 37°C and 250 rpm in an Innova 4230 Incubator Shaker (New Brunswick Scientific, Edison, NJ) for eight to nine hours. This culture (3 mL) was used to start an anaerobic fermentation. Two 100 mL anaerobic cultures of ALS(DE3)/pET11a and ALS(DE3)/pEIITHrEI were grown in serum bottles using LB media supplemented with 0.4% glucose, 50 μ g/mL
15 carbenicillin, and 100 mM MOPS. The cultures were grown overnight at 37°C without shaking. The overnight grown cultures were sub-cultured in serum bottles using fresh LB media supplemented with 0.4% glucose, 50 μ g/mL carbenicillin, and 100 mM MOPS. The starting OD(600) of these cultures was adjusted to 0.3. These serum bottles were incubated at 37°C without shaking. After one hour of incubation, the cultures were
20 induced with 100 μ M IPTG. A 3 mL sample was taken from each of the serum bottles at 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, and 24 hours. The samples were transferred into two properly labeled 2 mL microcentrifuge tubes, each containing 1.5 mL sample. The samples were spun down in a microcentrifuge centrifuge at 14000 g for 3 minutes. The supernatant was passed through a 0.45 μ syringe filter, and the resulting
25 filtrate was stored at -20°C until further analysis. The formation of fermentation products, mainly lactate and 3-hydroxypropionate, was measured by detecting derivatized CoA esters of lactate and 3-HP using LC/MS.

The following methods were performed to convert lactate and 3-HP into their respective CoA esters. Briefly, the filtrates were mixed with CoA-reaction buffer (200
30 mM potassium phosphate buffer, 2 mM acetyl-CoA, and 0.1 mg/mL purified transferase) in 1:1 ratio. The reaction was allowed to proceed for 20 minutes at room temperature.

The reaction was stopped by adding 1 volume of 10% TFA. The sample was purified using Sep Pak Vac columns (Waters). The column was conditioned with methanol and washed two times with 0.1% TFA. The sample was then applied to the column, and the column was washed two more times with 0.1% TFA. The sample was eluted with 40% acetonitrile, 0.1% TFA. The acetonitrile was removed from the sample by vacuum centrifugation. The samples were then analyzed by LC/MS.

Analysis of the standard CoA/CoA thioester mixtures and the CoA thioester mixtures derived from fermentation broths were carried out using a Waters/Micromass ZQ LC/MS instrument which had a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and the single quadrupole mass spectrometer. LC separations were made using a 4.6 x 150 mm YMC ODS-AQ (3 μ m particles, 120 Å pores) reversed-phase chromatography column at room temperature. Two gradient elution systems were developed using different mobile phases for the separation of the CoA esters. These two systems are summarized in Table 3. Elution system 1 was developed to provide the most rapid and efficient separation of the five-component CoA/CoA thioester mixture (CoA, acetyl-CoA, lactyl-CoA, acrylyl-CoA, and propionyl-CoA), while elution system 2 was developed to provide baseline separation of the structurally isomeric esters lactyl-CoA and 3HP-CoA in addition to separation of the remaining esters listed above. In all cases, the flow rate was 0.250 mL/minute, and photodiode array UV absorbance was monitored from 200 nm to 400 nm. All parameters of the electrospray MS system were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest and production of characteristic fragment ions. The following instrumental parameters were used for ESI-MS detection of CoA and organic acid-CoA thioesters in the positive ion mode: Capillary: 4.0 V; Cone: 56 V; Extractor: 1 V; RF lens: 0 V; Source temperature: 100°C; Desolvation temperature: 300°C; Desolvation gas: 500 L/hour; Cone gas: 40 L/hour; Low mass resolution: 13.0; High mass resolution: 14.5; Ion energy: 0.5; Multiplier: 650. Uncertainties for reported mass/charge ratios (m/z) and molecular masses are $\pm 0.01\%$. Table 3 provides a summary of gradient elution systems for the separation of organic acid-Coenzyme A thioesters.

Table 3

System	Buffer A	Buffer B	Gradient	
			Time	Percent B
1	25 mM ammonium acetate 0.5 % acetic acid	ACN 0.5 % acetic acid	0	10
			40	40
			42	100
			47	100
			50	10
2	25 mM ammonium acetate 10 mM TEA 0.5 % acetic acid	ACN 0.5 % acetic acid	0	10
			10	10
			45	60
			50	100
			53	100
			54	10

The following methods were used to separate the derivatized 3-hydroxypropionyl-CoA, which was formed from 3-HP, from 2-hydroxypropionyl-CoA (i.e., lactyl-CoA), which was formed from lactate. Because these structural isomers have identical masses and mass spectral fragmentation behavior, the separation and identification of these analytes in a mixture depends on their chromatographic separation. While elution system 1 provided excellent separation of the CoA thioesters tested (Figure 46), it was unable to resolve 3-HP-CoA and lactyl-CoA. An alternative LC elution system was developed using ammonium acetate and triethylamine (system 2; Table 3).

The ability of system 2 to separate 3-HP-CoA and lactyl-CoA was tested on a mixture of these two compounds. Comparing the results from a mixture of 3-HP-CoA and lactyl-CoA (Figure 47, Panel A) to the results from lactyl-CoA only (Figure 47, Panel B) revealed that system 2 can separate 3-HP-CoA and lactyl-CoA. The mass spectrum recorded under peak 1 (Figure 47, Panel A insert) was used to identify peak 1 as being a hydroxypropionyl-CoA thioester when compared to Figure 46, Panel C. In addition, comparison of Panels A and B of Figure 47 as well as the mass spectra results

corresponding to each peak revealed that peak 1 corresponds to 3-HP-CoA and peak 2 corresponds to lactyl-CoA.

System 2 was used to confirm that *E. coli* transfected with pEIITHrEI produced 3-HP in that 3-HP-CoA was detected. Specifically, an ion chromatogram for $m/z = 840$ in the analysis of a CoA transferase-treated fermentation broth aliquot collected from a culture of *E. coli* containing pEIITHrEI revealed the presence of 3-HP-CoA (Figure 48, Panel A). The CoA transferase-treated fermentation broth aliquot collected from a culture of *E. coli* lacking pEIITHrEI did not exhibit the peak corresponding to 3-HP-CoA (Figure 48, Panel B). Thus, these results indicate that the pEIITHrEI plasmid directs the expression of polypeptides having propionyl-CoA transferase activity, lactyl-CoA dehydratase activity, and acrylyl-CoA hydratase activity. These results also indicate that expression of these polypeptides leads to the formation of 3-HP.

**Example 9 – Cloning nucleic acid molecules that encode
a polypeptide having acetyl CoA carboxylase activity**

Polypeptides having acetyl-CoA carboxylase activity catalyze the first committed step of the fatty acid synthesis by carboxylation of acetyl-CoA to malonyl-CoA.

Polypeptides having acetyl-CoA carboxylase activity are also responsible for providing malonyl-CoA for the biosynthesis of very-long-chain fatty acids required for proper cell function. Polypeptides having acetyl-CoA carboxylase activity can be biotin dependent enzymes in which the cofactor biotin is post-translationally attached to a specific lysine residue. The reaction catalyzed by such polypeptides consists of two discrete half reactions. In the first half reaction, biotin is carboxylated by biocarbonate in an ATP-dependent reaction to form carboxybiotin. In the second half reaction, the carboxyl group is transferred to acetyl-CoA to form malonyl-CoA.

Prokaryotic and eukaryotic polypeptides having acetyl-CoA carboxylase activity exist. The prokaryotic polypeptide is a multi-subunit enzyme (four subunits), where each of the subunits is encoded by a different nucleic acid sequence. For example, in *E. coli*, the following genes encode for the four subunits of acetyl-CoA carboxylase:

accA: Acetyl-coenzyme a carboxylase carboxyl transferase subunit alpha
(GenBank® accession number M96394)

accB: Biotin carboxyl carrier protein (GenBank® accession number U18997)

accC: Biotin carboxylase (GenBank® accession number U18997)

accD: Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (GenBank® accession number M68934)

5 The eukaryotic polypeptide is a high molecular weight multi-functional enzyme encoded by a single gene. For example, in *Saccharomyces cerevisiae*, the acetyl-CoA carboxylase can have the sequence set forth in GenBank® accession number M92156.

 The prokaryotic type acetyl-CoA carboxylase from *E. coli* was overexpressed using T7 promoter vector pFN476 as described elsewhere (Davis *et al. J. Biol. Chem.*,
10 275:28593-28598 (2000)). The eukaryotic type acetyl-CoA carboxylase gene was amplified from *Saccharomyces cerevisiae* genomic DNA. Two primers were designed to amplify the *acc1* gene from in *S. cerevisiae* (*acc1F* 5'-

 atagGCGGCCGCAGGAATGCTGTATGAGCGAAGAAAGCTTATT C-3', SEQ ID
 NO: 138 where the bold is homologous sequence, the italics is a *Not* I site, the underline
15 is a RBS, and the lowercase is extra; and *acc1R* 5'-atgctcgcatCTCGAGTAG-

 CTAAATTAAATTACATCAATAGTA-3', SEQ ID NO: 139 where the bold is
 homologous sequence, the italics is a *Xho* I site, and the lowercase is extra). The
 following PCR mix is used to amplify *acc1* gene 10X *pfu* buffer (10 µL), dNTP (10mM;
 2 µL), cDNA (2 µL), *acc1F* (100 µM; 1 µL), *acc1R* (100 µM; 1 µL), *pfu* enzyme (2.5

20 units/µL; 2 µL), and DI water (82 µL). The following protocol was used to amplify the
 acc1 gene. After performing PCR, the PCR product was separated on a gel, and the band
 corresponding to *acc1* nucleic acid (about 6.7 Kb) was gel isolated using Qiagen gel
 isolation kit. The PCR fragment is digested with *Not* I and *Xho* I (New England BioLab)

 restriction enzymes. The digested PCR fragment is then ligated to pET30a which was
25 restricted with *Not* I and *Xho* I and dephosphorylated with SAP enzyme. The *E. coli*
 strain DH10B was transformed with 1 µL of the ligation mix, and the cells were
 recovered in 1 mL of SOC media. The transformed cells were selected on LB/kanamycin
 (50 µg/µL) plates. Eight single colonies are selected, and PCR was used to screen for the
 correct insert. The plasmid having correct insert was isolated using Qiagen Spin Mini
30 prep kit.

To obtain a polypeptide having acetyl-CoA carboxylase activity, the plasmid pMSD8 or pET30a/accl overexpressing *E. coli* or *S. cerevisiae* acetyl-CoA carboxylase was transformed into Tuner pLacI chemically competent cells (Novagen, Madison, WI). The transformed cells were selected on LB/chloramphenicol (25 µg/mL) plus carbencillin (50 µg/mL) or kanamycin (50 µg/mL).

A crude extract of this strain can be prepared in the following manner. An overnight culture of Tuner pLacI with pMSD8 is subcultured into 200 mL (in one liter baffle culture flask) of fresh M9 media supplemented with 0.4% glucose, 1 µg/mL thiamine, 0.1% casamino acids, and 50 µg/mL carbencillin or 50 µg/mL kanamycin and 25 µg/mL chloramphenicol. The culture is grown at 37°C in a shaker with 250 rpm agitation until it reaches an optical density at 600 nm of about 0.6. IPTG is then added to a final concentration of 100 µM. The culture is then incubated for an additional 3 hours with shaking speed of 250 rpm at 37°C. Cells are harvested by centrifugation at 8000 x g and are washed one time with 0.85% NaCl. The cell pellet was resuspended in a minimal volume of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, and 5% glycerol. The cells are lysed by passing them two times through a French Pressure cell at 1000 psig pressure. The cell debris was removed by centrifugation for 20 minutes at 30,000 x g.

The enzyme can be assayed using a method from Davis *et al.* (*J. Biol. Chem.*, 275:28593-28598 (2000)).

Example 10 – Cloning a nucleic acid molecule that encodes a polypeptide having malonyl-CoA reductase activity from *Chloroflexus auarantiacus*

A polypeptide having malonyl-CoA reductase activity was partially purified from *Chloroflexus auarantiacus* and used to obtain amino acid micro-sequencing results. The amino acid sequencing results were used to identify and clone the nucleic acid that encodes a polypeptide having malonyl-CoA reductase activity.

Biomass required for protein purification was grown in B. Braun BIOSTAT B fermenters (B. Braun Biotech International GmbH, Melsungen, Germany). A glass vessel fitted with a water jacket for heating was used to grow the required biomass. The glass

vessel was connected to its own control unit. The liquid working volume was 4 L, and the fermenter was operated at 55°C with 75 rpm of agitation. Carbon dioxide was occasionally bubbled through the headspace of the fermenter to maintain anaerobic conditions. The pH of the cultures was monitored using a standard pH probe and was maintained between 8.0 and 8.3. The inoculum for the fermenters was grown in two 250 mL bottles in an Innova 4230 Incubator, Shaker (New Brunswick Scientific, Edison, NJ) at 55°C with interior lights. The fermenters were illuminated by three 65 W plant light reflector lamps (General Electric, Cleveland, OH). Each lamp was placed approximately 50 cm away from the glass vessel. The media used for the inoculum and the fermenter culture was as follows per liter: 0.07 g EDTA, 1 mL micronutrient solution, 1 mL FeCl₃ solution, 0.06 g CaSO₄·2 H₂O, 0.1 g MgSO₄·7 H₂O, 0.008 g NaCl, 0.075 g KCl, 0.103 g KNO₃, 0.68 g NaNO₃, 0.111 g Na₂HPO₄, 0.2 g NH₄Cl, 1 g yeast extract, 2.5 g casamino acid, 0.5 g Glycyl-Glycine, and 900 mL DI water. The micronutrient solution contained the following per liter: 0.5 mL H₂SO₄ (conc.), 2.28 g MnSO₄·7 H₂O, 0.5 g ZnSO₄·7 H₂O, 0.5 g H₃BO₃, 0.025 g CuSO₄·2 H₂O, 0.025 g Na₂MoO₄·2 H₂O, and 0.045 g CoCl₂·6 H₂O. The FeCl₃ solution contained 0.2905 g FeCl₃ per liter. After adjusting the pH of the media to 8.2 to 8.4, 0.75 g/L Na₂S·9H₂O was added, the pH was readjusted to 8.2 to 8.4, and the media was filter-sterilized through a 0.22 µ filter.

The fermenter was inoculated with 500 mL of grown culture. The fermentation was stopped, and the biomass was harvested after the cell density was about 0.5 to 0.6 units at 600 nm.

The cells were harvested by centrifugation at 5000 x g (Beckman JLA 8.1000 rotor) at 4°C, washed with 1 volume of ice cold 0.85% NaCl, and centrifuged again. The cell pellet was resuspended in 30 mL of ice cold 100 mM Tris-HCl (pH 7.8) buffer that was supplemented with 2 mM DTT, 5 mM MgCl₂, 0.4 mM PEFABLOC (Roche Molecular Biochemicals, Indianapolis, IN), 1% streptomycin sulfate, and 2 tablets of Complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). The cell suspension was lysed by passing the suspension, three times, through a 50 mL French Pressure Cell operated at 1600 psi (gauge reading). Cell debris was removed by centrifugation at 30,000 x g (Beckman JA 25.50 rotor). The crude extract was filtered prior to chromatography using a 0.2 µm HT Tuffryn membrane

syringe filter (Pall Corp., Ann Arbor, MI). The protein concentration of the crude extract was 29 mg/mL, which was determined using the BioRad Protein Assay according to the manufacturer's microassay protocol. Bovine gamma globulin was used for the standard curve determination. This assay was based on the Bradford dye-binding procedure
5 (Bradford, *Anal. Biochem.*, 72:248 (1976)).

Before starting the protein purification, the following assay was used to determine the activity of malonyl-CoA reductase in the crude extract. A 50 μ L aliquot of the cell extract (29 mg/mL) was added to 10 μ L 1M Tris-HCl (final concentration in assay 100 mM), 10 μ L 10 mM malonyl CoA (final concentration in assay 1 mM), 5.5 μ L 5.5 mM
10 NADPH (final concentration in assay 0.3 mM), and 24.5 μ L DI water in a 96 well UV transparent plate (Corning, NY). The enzyme activity was measured at 45°C using SpectraMAX Plus 96 well plate reader (Molecular devices Sunnyvale, CA). The activity of malonyl-CoA reductase was monitored by measuring the disappearance of NADPH at 340 nm wavelength. ~~The crude extract exhibited malonyl-CoA reductase activity.~~

15 The 5 mL (total 145 mg) protein cell extract was diluted with 20 mL buffer A (20 mM ethanolamine (pH 9.0), 5 mM $MgCl_2$, 2 mM DTT). The chromatographic protein purification was conducted using a BioLogic protein purification system (BioRad Hercules, CA). ~~The 25 mL of cell suspension was loaded onto a UNO Q-6 ion-exchange column that had been equilibrated with buffer A at a rate of 1 mL/minute. After sample~~
20 ~~loading, the column was washed with 2.5 times column volume of buffer A at a rate of 2 mL/minute. The proteins were eluted with a linear gradient of NaCl in buffer A from 0-0.33 M in 25 Column volume. During the entire chromatographic separation, three mL~~ fractions were collected. The collection tubes contained 50 μ L of Tris-HCl (pH 6.5) so that the pH of the eluted sample dropped to about pH 7. Major chromatographic peaks
25 were detected in the region that corresponded to fractions 18 to 21 and 23 to 30. A 200 μ L sample was taken from these fractions and concentrated in a microcentrifuge at 4°C using a Microcon YM-10 columns (Millipore Corp., Bedford, MA) as per manufacture's instructions. To each of the concentrated fraction, buffer A-Tris (100 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 2 mM DTT) was added to bring the total volume to 100 μ L. Each of
30 these fractions was tested for the malonyl-CoA reductase activity using the spectrophotometric assay described above. The majority of specific malonyl CoA activity

was found in fractions 18 to 21. These fractions were pooled together, and the pooled sample was desalted using PD-10 column (Amersham Pharmacia Piscataway, NJ) as per manufacture's instructions.

The 10.5 mL of desalted protein extract was diluted with buffer A-Tris to a volume of 25 mL. This desalted diluted sample was applied to a 1 mL HiTrap Blue column (Amersham Pharmacia Piscataway, NJ) which was equilibrated with buffer A-Tris. The sample was loaded at a rate of 0.1 mL/minute. Unbound proteins were washed with 2.5 CV buffer A-Tris. The protein was eluted with 100 mM Tris (pH 7.8), 5 mM $MgCl_2$, 2 mM DTT, 2mM NADPH, and 1 M NaCl. During this separation process, one mL fractions were collected. A 200 μ L sample was drawn from fractions 49 to 54 and concentrated. Buffer A-Tris was added to each of the concentrated fractions to bring the total volume to 100 μ L. Fractions were assayed for enzyme activity as described above. The highest specific activity was observed in fraction 51. The entire fraction 51 was concentrated as described above, and the concentrated sample was separated on an SDS-PAGE gel.

Electrophoresis was carried out using a Bio-Rad Protean II minigel system and pre-cast SDS-PAGE gels (4-15%), or a Protean II XI system and 16 cm x 20 cm x 1mm SDS-PAGE gels (10%) cast as per the manufacturer's protocol. The gels were run according to the manufacturer's instructions with a running buffer of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% SDS.

A gel thickness of 1 mm was used to run samples from fraction 51. Protein from fraction 51 was loaded onto 10% SDS-PAGE (3 lanes, each containing 75 μ g of total protein). The gels were stained briefly with Coomassie blue (Bio-Rad, Hercules, CA) and then destained to a clear background with a 10% acetic acid and 20% methanol solution. The staining revealed a band of about 130 to 140 KDa.

The protein band of about 130-140 KDa was excised with no excess unstained gel present. An equal area gel without protein was excised as a negative control. The gel slices were placed in uncolored microcentrifuge tubes, prewashed with 50% acetonitrile in HPLC-grade water, washed twice with 50% acetonitrile, and shipped on dry ice to Harvard Microchemistry Sequencing Facility, Cambridge, MA.

After *in-situ* enzymatic digestion of the polypeptide sample with trypsin, the resulting polypeptides were separated by micro-capillary reverse-phase HPLC. The HPLC was directly coupled to the nano-electrospray ionization source of a Finnigan LCQ quadrupole ion trap mass spectrometer (μ LC/MS/MS). Individual sequence spectra
5 (MS/MS) were acquired on-line at high sensitivity for the multiple polypeptides separated during the chromatographic run. The MS/MS spectra of the polypeptides were correlated with known sequences using the algorithm Sequest developed at the University of Washington (Eng *et al.*, *J. Am. Soc. Mass Spectrom.*, 5:976 (1994)) and programs developed at Harvard (Chittum *et al.*, *Biochemistry*, 37:10866 (1998)). The results were
10 reviewed for consensus with known proteins and for manual confirmation of fidelity.

A similar purification procedure was used to obtain another sample (protein 1 sample) that was subjected to the same analysis that was used to evaluate the fraction 51 sample.

The polypeptide sequence results indicated that the polypeptides obtained from
15 both the fraction 51 sample and the protein 1 sample had similarity to the six (764, 799, 859, 923, 1090, 1024) contigs sequenced from the *C. aurantiacus* genome and presented on the Joint Genome Institute's web site (<http://www.jgi.doe.gov/>). The 764 contig was the most prominent of the six with about 40 peptide sequences showing similarity. BLASTX analysis of each of these contigs on the GenBank web site
20 (<http://www.ncbi.nlm.nih.gov/BLAST/>) indicated that the DNA sequence of the 764 contig (4201 bases) encoded for polypeptides that had a dehydrogenase/reductase type activity. Close inspection of the 764 contig, however, revealed that this contig did not have an appropriate ORF that would encode for a 130-140 KDa polypeptide.

BASLTX analysis also was conducted using the other five contigs. The results of
25 this analysis were as follows. The 799 contig (3173 bases) appeared to encode polypeptides having phosphate and dehydrogenase type activities. The 859 contig (5865 bases) appeared to encode polypeptides having synthetase type activities. The 923 contig (5660 bases) appeared to encode polypeptides having elongation factor and synthetase type activities. The 1090 contig (15201 bases) appeared to encode polypeptides having
30 dehydrogenase/reductase and cytochrome and sigma factor activities. The 1024 contig (12276 bases) appeared to encode polypeptides having dehydrogenase and decarboxylase

and synthetase type activities. Thus, the 859 and 923 contigs were eliminated from any further analysis.

The results from the BLASTX analysis also revealed that the dehydrogenase found in the 1024 contig was most likely an inositol monophosphate dehydrogenase.

- 5 Thus, the 1024 contig was eliminated as a possible candidate that might encode for a polypeptide having malonyl-CoA reductase activity. The 799 contig also was eliminated since this contig is part of the OS17 polypeptide described above.

- This narrowed down the search to 2 contigs, the 764 and 1090 contigs. Since the contigs were identified using the same protein sample and the dehydrogenase activities
10 found in these contigs gave very similar BLASTX results, it was hypothesized that they are part of the same polypeptide. Additional evidence supporting this hypothesis was obtained from the discovery that the 764 and 1090 contigs are adjacent to each other in the *C. aurantiacus* genome as revealed by an analysis of scaffold data provided by the Joint Genome Institute. Sequence similarity and assembly analysis, however, revealed no
15 overlapping sequence between these two contigs, possibly due to the presence of gaps in the genome sequencing.

- The polypeptide sequences that belonged to the 764 and 1090 contigs were mapped. Based on this analysis, an appropriate coding frame and potential start and stop codons were identified. The following PCR primers were designed to PCR amplify a
20 fragment that encoded for a polypeptide having malonyl-CoA reductase activity: PRO140F 5'-ATGGCGACGGGCGAGTCCATGAG-3', SEQ ID NO:153; PRO140R 5'-GGACACGAAGAACAGGGCGACAC-3', SEQ ID NO:154; and PRO140UP 5'-GAACTGTCTGGAGTAAGGCTGTC-3', SEQ ID NO:155. The PRO140F primer was designed based on the sequence of the 1090 contig and corresponds to the start of the
25 potential start codon. The twelfth base was change from G to C to avoid primer-dimer formation. This change does not change the amino acid that was encoded by the codon. The PRO140R primer was designed based on sequence of the 764 contig and corresponds to a region located about 1 kB downstream from the potential stop codon. The PRO140UPF primer was designed based on sequence of the 1090 contig and corresponds
30 to a region located about 300 bases upstream of potential start codon.

Genomic *C. aurantiacus* DNA was obtained. Briefly, *C. aurantiacus* was grown in 50 mL cultures for 3 to 4 days. Cells were pelleted and washed with 5 mL of a 10 mM Tris solution. The genomic DNA was then isolated using the gram positive bacteria protocol provided with Gentra Genomic "Puregene" DNA isolation kit (Gentra Systems, Minneapolis, MN). The cell pellet was resuspended in 1 mL Gentra Cell Suspension Solution to which 14.2 mg of lysozyme and 4 μ L of 20 mg/mL proteinase K solution was added. The cell suspension was incubated at 37°C for 30 minutes. The precipitated genomic DNA was recovered by centrifugation at 3500g for 25 minutes and air-dried for 10 minutes. The genomic DNA was suspended in an appropriate amount of a 10 mM Tris solution and stored at 4°C.

Two PCR reactions were set-up using *C. aurantiacus* genomic DNA as template as follows:

PCR Reaction #1			PCR program
15	3.3 X <i>rTH</i> polymerase Buffer	30 μ L	94°C 2 minutes
	Mg(OAC) (25 mM)	4 μ L	29 cycles of:
	dNTP Mix (10 mM)	3 μ L	94°C 30 seconds
	PRO140F (100 μ M)	2 μ L	63°C 45 seconds
	PRO140R (100 μ M)	2 μ L	68°C 4.5 minutes
20	Genomic DNA (100 ng/mL)	1 μ L	68°C 7 minutes
	<i>rTH</i> polymerase (2 U/ μ L)	2 μ L	4°C Until further use
	<i>pfu</i> polymerase (2.5 U/ μ L)	0.25 μ L	
	DI water	55.75 μ L	
	Total	100 μ L	
25	PCR Reaction #2		PCR program
	3.3 X <i>rTH</i> polymerase Buffer	30 μ L	94°C 2 minutes
	Mg(OAC) (25 mM)	4 μ L	29 cycles of:
	dNTP Mix (10 mM)	3 μ L	94°C 30 seconds
30	PRO140UPF (100 μ M)	2 μ L	60°C 45 seconds
	PRO140R (100 μ M)	2 μ L	68°C 4.5 minutes

Genomic DNA (100 ng/mL)	1 μ L	68°C	7 minutes
<i>rTH</i> polymerase (2 U/ μ L)	2 μ L	4°C	Until further use
<i>pfu</i> polymerase 2.5 U/ μ L)	0.25 μ L		
DI water	55.75 μ L		
5 Total	100 μ L		

The products from both PCR reactions were separated on a 0.8% TAE gel. Both PCR reactions produced a product of 4.7 to 5 Kb in size. This approximately matched the expected size of a nucleic acid molecule that could encode a polypeptide having malonyl-CoA reductase activity.

Both PCR products were sequenced using sequencing primers (1090Fseq 5'-GATTCCGTATGTCACCCCTA-3', SEQ ID NO:156; and 764Rseq 5'-CAGGCGACTGGCAATCACAA-3', SEQ ID NO:157). The sequence analysis revealed a gap between the 764 and 1090 contigs. The nucleic acid sequence between the sequences from the 764 and 1090 contigs was greater than 300 base pairs in length (Figure 51). In addition, the sequence analysis revealed an ORF of 3678 bases that showed similarities to dehydrogenase/reductase type enzymes (Figure 52). The amino acid sequence encoded by this ORF is 1225 amino acids in length (Figure 50). Also, BLASTP analysis of the amino acid sequence encoded by this ORF revealed two short chain dehydrogenase domains (adh type). These results are consistent with a polypeptide having malonyl-CoA reductase activity since such an enzyme involves two reduction steps for the conversion of malonyl CoA to 3-HP. Further, the computed MW of the polypeptide was determined to be about 134 KDa.

PCR was conducted using the PRO140F/PRO140R primer pair, *C. aurantiacus* genomic DNA, and the protocol described above as PCR reaction #1. After the PCR was completed, 0.25 U of *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN) was added to the PCR mix, which was then incubated at 72°C for 10 minutes. The PCR product was column purified using Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The purified PCR product was then TOPO cloned into expression vector pCRT7/CT as per manufacture's instructions (Invitrogen, Carlsbad, CA). TOP10 F' chemical-competent cells were transformed with the TOPO ligation mix as per

manufacture's instructions (Invitrogen, Carlsbad, CA). The cells were recovered for half an hour, and the transformants were selected on LB/ampicillin (100 µg/mL) plates. Twenty single colonies were selected, and the plasmid DNA was isolated using Qiagen spin Mini prep kit (Qiagen Inc., Valencia, CA).

- 5 Each of these twenty clones were tested for correct orientation and right insert-size by PCR. Briefly, plasmid DNA was used as a template, and the following two primers were used in the PCR amplification: PCRT7 5'-GAGACCACAACGGTTTCCCTCTA-3', SEQ ID NO:158; and PRO140R 5'-GGACACGAAGAACAGGGCGACAC-3', SEQ ID NO:159. The following PCR reaction mix and program was used:

10

PCR Reaction		PCR program
3.3 X <i>rTH</i> polymerase Buffer	7.5 µL	94°C 2 minutes
Mg(OAC) (25 mM)	1 µL	25 cycles of:
15 dNTP Mix (10 mM)	0.5 µL	94°C 30 seconds
PCRT7 (100 µM)	0.125 µL	55°C 45 seconds
PRO140R (100 µM)	0.125 µL	68°C 4 minutes
Plasmid DNA	0.5 µL	68°C 7 minutes
<i>rTH</i> polymerase (2 U/µL)	0.5 µL	4°C Until further use
20 DI water	14.75 µL	
Total	25 µL	

- Out of twenty clone tested, only one clone exhibited the correct insert (Clone # P-10). Chemical competent cells of BL21(DE3)pLysS (Invitrogen, Carlsbad, CA) were transformed with 2 µL of the P-10 plasmid DNA as per the manufacture's instructions. The cells were recovered at 37°C for 30 minutes and were plated on LB ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL).

- A 20 mL culture of BL21(DE3)pLysS/P-10 and a 20 mL control culture of BL21(DE3)pLysS was incubated overnight. Using the overnight cultures as an inoculum, two 100 mL BL21(DE3)pLysS/P-10 clone cultures and two control strain cultures (BL21(DE3)pLysS) were started. All the cultures were induced with IPTG when they

reached an OD of about 0.5 at 600 nm. The control strain culture was induced with 10 μ M IPTG or 100 μ M IPTG, while one of the BL21(DE3)pLysS/P-10 clone cultures was induced with 10 μ M IPTG and the other with 100 μ M IPTG. The cultures were grown for 2.5 hours after induction. Aliquots were taken from each of the culture flasks before and
5 after 2.5 hours of induction and separated using 4-15% SDS-PAGE to analyze polypeptide expression. In the induced BL21(DE3)pLysS/P-10 samples, a band corresponding to a polypeptide having a molecular weight of about 135 KDa was observed. This band was absent in the control strain samples and in samples taken before IPTG induction.

10 To assess malonyl-CoA reductase activity, BL21(DE3)pLysS/P-10 and BL21(DE3)pLysS cells were cultured and then harvested by centrifugation at 8,000 x g (Rotor JA 16.250, Beckman Coulter, Fullerton, CA). Once harvested, the cells were washed once with an equal volume of a 0.85% NaCl solution. The cell pellets were resuspended into 100 mM Tris-HCl buffer that was supplemented with 5 mM Mg_2Cl and
15 2 mM DTT. The cells were disrupted by passing twice through a French Press Cell at 1,000 psi pressure (Gauge value). The cell debris was removed by centrifugation at 30,000 x g (Rotor JA 25.50, Beckman Coulter, Fullerton, CA). The cell extract was maintained at 4°C or on ice until further use.

Activity of malonyl-CoA reductase was measured at 37°C for both the control
20 cells and the IPTG-induced cells. The activity of malonyl-CoA reductase was monitored by observing the disappearance of added NADPH as described above. No activity was found in the cell extract of the control strain, while the cell extract from the IPTG-induced BL21(DE3)pLysS/P-10 cells displayed malonyl-CoA reductase activity with a specific activity calculated to be about 0.0942 μ mole/minute/mg of total protein.

25 Malonyl-CoA reductase activity also was measured by analyzing 3-HP formation from malonyl CoA using the following reaction conducted at 37°C:

	Volume	Final conc.
Tris HCl (1M)	10 μ L	100mM
Malonyl CoA (10mM)	40 μ L	4 mM
30 NADPH (10 mM)	30 μ L	3 mM
Cell-extract	20 μ L	

Total 100 μ L

The reaction was carried out at 37°C for 30 minutes. In the control reaction, a cell extract from BL21(DE3)pLysS was added to a final concentration of 322 mg total protein. In the experimental reaction mix, a cell extract from BL21(DE3)pLysS/P-10 was added to a final concentration of 226 mg of total protein. The reaction mixtures were frozen at -20°C until further analysis.

Chromatographic separation of the components in the reaction mixtures was performed using a HPX-87H (7.8x300mm) organic acid HPLC column (BioRad Laboratories, Hercules, CA). The column was maintained at 60°C. Mobile phase composition was HPLC grade water pH to 2.5 using trifluoroacetic acid (TFA) and was delivered at a flow rate of 0.6 mL/minute.

Detection of 3-HP in the reaction samples was accomplished using a Waters/Micromass ZQ LC/MS instrument consisting of a Waters 2690 liquid chromatograph (Waters Corp., Milford, MA) with a Waters 996 Photo-diode Array (PDA) absorbance monitor placed in series between the chromatograph and the single quadrupole mass spectrometer. The ionization source was an Atmospheric Pressure Chemical Ionization (APCI) ionization source. All parameters of the APCI-MS system were optimized and selected based on the generation of the protonated molecular ion $[M+H]^+$ of 3-HP. The following parameters were used to detect 3-HP in the positive ion mode: Corona: 10 μ A; Cone: 20V; Extractor: 2V; RF lens: 0.2V; Source temperature: 100°C; APCI Probe temperature: 300°C; Desolvation gas: 500L/hour; Cone gas: 50L/hour; Low mass resolution: 15; High mass resolution: 15; Ion energy: 1.0; Multiplier: 650. Data was collected in Selected Ion Reporting (SIR) mode set at $m/z = 90.9$.

Both the control reaction sample and the experimental reaction sample were probed for presence of 3-HP using the HPLC-mass spectroscopy technique. In the control samples, no 3-HP peak was observed, while the experimental sample exhibited a peak that matched the retention and the mass of 3-HP.

Example 11 – Constructing recombinant cells that produce 3-HP

A pathway to make 3-hydroxypropionate directly from glucose via acetyl CoA is presented in Figure 44. Most organisms such as *E. coli*, *Bacillus*, and yeast produce acetyl CoA from glucose via glycolysis and the action of pyruvate dehydrogenase. In order to divert the acetyl CoA generated from glucose, it is desirable to overexpress two genes, one encoding for acetyl CoA carboxylase and the other encoding malonyl-CoA reductase. As an example, these genes are expressed in *E. coli* through a T7 promoter using vectors pET30a and pFN476. The vector pET30a has a pBR *ori* and kanamycin resistance, while pFN476 has pSC101 *ori* and uses carbencillin resistance for selection. Because these two vectors have compatible *ori* and different markers they can be maintained in *E. coli* at the same time. Hence, the constructs used to engineer *E. coli* for direct production of 3-hydroxypropionate from glucose are pMSD8 (pFN476/accABCD) (Davis *et al.*, *J. Biol. Chem.*, 275:28593-28598, 2000) and pET30a/malonyl-CoA reductase or pET30a/*acc1* and pFN476/malonyl-CoA reductase. The constructs are depicted in Figure 45.

To test the production of 3-hydroxypropionate from glucose, *E. coli* strain Tuner pLacI carrying plasmid pMSD8 (pFN476/accABCD) and pET30a/malonyl-CoA reductase or pET30a/*acc1* and pFN476/malonyl-CoA reductase are grown in a B. Braun BIOSTAT B fermenter. A glass vessel fitted with a water jacket for heating is used to conduct this experiment. The fermenter working volume is 1.5 L and is operated at 37°C. The fermenter is continuously supplied with oxygen by bubbling sterile air through it at a rate of 1 vvm. The agitation is cascaded to the dissolve oxygen concentration which is maintained at 40% DO. The pH of the liquid media is maintained at 7 using 2 N NaOH. The *E. coli* strain is grown in M9 media supplemented with 1% glucose, 1 µg/mL thiamine, 0.1% casamino acids, 10 µg/mL biotin, 50 µg/mL carbencillin, 50 µg/mL kanamycin, and 25 µg/mL chloramphenicol. The expression of the genes is induced when the cell density reached 0.5 OD(600nm) by adding 100 µM IPTG. After induction, samples of 2 mL volume are taken at 1, 2, 3, 4, and 8 hours. In addition, at 3 hours after induction, a 200 mL sample is taken to make a cell extract. The 2 mL samples are spun, and the supernatant is used to analyze products using LC/MS technique. The supernatant is stored at -20°C until further analysis.

The extract is prepared by spinning the 200 mL of cell suspension at 8000 g and washing the cell pellet with 50 mL of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, and 5% glycerol. The cell suspension is spun again at 8000 g, and the pellet is resuspended into 5 mL of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, and 5% glycerol. The cells are disrupted by passing twice through a French Press at 1000 p.s.g. The cell debris is removed by centrifugation for 20 minutes at 30,000 g. All the operations are conducted at 4°C. To demonstrate *in vitro* formation of 3-hydroxypropionate using this recombinant cell extract, the following reaction of 200 µL is conducted at 37°C. The reaction mix is as follows: Tris HCl (pH 8.0; 100 mM), ATP (1 mM), MgCl₂ (5 mM), KCl (100 mM), DTT (5 mM), NaHCO₃ (40 mM), NADPH (0.5 mM), acetyl CoA (0.5 mM), and cell extract (0.2 mg). The reaction is stopped after 15 minutes by adding 1 volume of 10% trifluoroacetic acid (TFA). The products of this reaction are detected using an LC/MS technique.

The detection and analysis for the presence of 3-hydroxypropionate in the supernatant and the *in vitro* reaction mixture is carried out using a Waters/Micromass ZQ LC/MS instrument. This instrument consists of a Waters 2690 liquid chromatograph with a Waters 2410 refractive index detector placed in series between the chromatograph and the single quadrupole mass spectrometer. LC separations are made using a Bio-Rad Aminex 87-H ion-exchange column at 45°C. Sugars, alcohol, and organic acid products are eluted with 0.015% TFA buffer. For elution, the buffer is passed at a flow rate of 0.6 mL/minute. For detection and quantification of 3-hydroxypropionate, a sample obtained from TCI, America (Portland, OR) is used as a standard.

Example 12 Cloning of propionyl-CoA transferase, lactyl-CoA dehydratase (LDH), and a hydratase (OS19) for Expression in *Saccharomyces cerevisiae*

The pESC Yeast Epitope Tagging Vector System (Stratagene, La Jolla, CA) was used in cloning the genes involved in 3-hydroxypropionic acid production via lactic acid. The pESC vectors each contain GAL1 and GAL10 promoters in opposing directions, allowing the expression of two genes from each vector. The GAL1 and GAL10 promoters are repressed by glucose and induced by galactose. Each of the four available pESC vectors has a different yeast-selectable marker (HIS3, TRP1, LEU2, URA3) so

- multiple plasmids can be maintained in a single strain. Each cloning region has a polylinker site for gene insertion, a transcription terminator, and an epitope coding sequence for C-terminal or N-terminal epitope tagging of expressed proteins. The pESC vectors also have a ColE1 origin of replication and an ampicillin resistance gene to allow
- 5 replication and selection in *E. coli*. The following vector/promoter/nucleic acid combinations were constructed:

Vector	Promoter	Polypeptide	Source of nucleic acid
pESC-Trp	GAL1	OS19 hydratase	<i>Chloroflexus aurantiacus</i>
	GAL10	E1	<i>Megasphaera elsdenii</i>
pESC-Leu	GAL1	E2 α	<i>Megasphaera elsdenii</i>
	GAL10	E2 β	<i>Megasphaera elsdenii</i>
pESC-His	GAL1	D-LDH	<i>Escherichia coli</i>
	GAL10	PCT	<i>Megasphaera elsdenii</i>

The primers used were as follows:

- 10 OS19APAF: 5'-ATAGGGCCCAGGAGATCAAACCATGGGTGAAGAGTCT-
CTGGTTC-3' (SEQ ID NO:164)
- OS19SALR: 5'-CCTCTGCTACAGTCGACACAACGACCACTGAAGTTG-
GGAG-3' (SEQ ID NO:165)
- OS19KPNR: 5'-AGTCTGCTATCGGTACCTCAACGACCACTGAAGTTG-
15 GGAG-3' (SEQ ID NO:166)
- EINOTF: 5'-ATAGCGGCCGCATAATGGATACTCTCGGAATCGACG-
TTGG-3' (SEQ ID NO:167)
- EICLAR: 5'-CCCCATCGATACATATTTCTTGATTTTATCATAAGCA-
ATC-3' (SEQ ID NO:168)
- 20 EII α APAF: 5'-CCAGGGCCCATAATGGGTGAAGAAAAACAGTAGA-
TATTG-3' (SEQ ID NO:169)
- EII α SALR: 5'-GGTAGACTTGTCGACGTAGTGGTTTCCTCCTTCATT-
GG-3' (SEQ ID NO:170)
- EII β NOTF: 5'-ATAGCGGCCGCATAATGGGTCAGATCGACGAACCTTA-

TCAG-3'(SEQ ID NO:171)

EII β SPER: 5'-AGGTTCAACTAGTTCGTAGAGGATTTCCGAGAAAGC-

CTG-3'(SEQ ID NO:172)

LDHAPAF: 5'-CTAGGGCCCATAATGGAACTCGCCGTTTATAG-

5 CAC-3'(SEQ ID NO:173)

LDHXHOR: 5'-ACTTCTCGAGTTAAACCAGTTCGTTCTGGGCA-

GGT-3'(SEQ ID NO:174)

PCTSPEF: 5'-GGGACTAGTATAATGGGAAAAGTAGAAATCAT-

TACAG-3'(SEQ ID NO:175)

10 PCTPACR: 5'-CGGCTTAATTAACAGCAGAGATTTATTTTTTCA-

GTCC-3'(SEQ ID NO:176)

All restriction enzymes were obtained from New England Biolabs, Beverly, MA. All plasmid DNA preparations were done using QIAprep Spin Miniprep Kits, and all gel purifications were done using QIAquick Gel Extraction Kits (Qiagen, Valencia, CA).

15

A. Construction of the pESC-Trp/OS19 hydratase vector

Two constructs in pESC-Trp were made for the OS19 nucleic acid from *C. aurantiacus*. One of these constructs utilized the *Apa* I and *Sal* I restriction sites of the GAL1 multiple cloning site and was designed to include the c-myc epitope. The second
20 construct utilized the *Apa* I and *Kpn* I sites and thus did not include the c-myc epitope sequence.

Six μ g of pESC-Trp vector DNA was digested with the restriction enzyme *Apa* I and the digest was purified using a QIAquick PCR Purification Column. Three μ g of the *Apa* I-digested vector DNA was then digested with the restriction enzyme *Kpn* I, and 3 μ g
25 was digested with *Sal* I. The double-digested vector DNAs were separated on a 1% TAE-agarose gel, purified, dephosphorylated with shrimp alkaline phosphatase (Roche Biochemical Products, Indianapolis, IN), and purified with a QIAquick PCR Purification Column.

The nucleic acid encoding the *Chloroflexus aurantiacus* polypeptide having
30 hydratase activity (OS19) was amplified from genomic DNA using the PCR primer pair OS19APAF and OS19SALR and the primer pair OS19APAF and OS19KPNR.

OS19APAF was designed to introduce an *Apa* I restriction site and a translation initiation site (ACCATGG) at the beginning of the amplified fragment. The OS19KPNR primer was designed to introduce a *Kpn* I restriction site at the end of the amplified fragment and to contain the translational stop codon for the hydratase gene. OS19SALR introduces a *Sal* I site at the end of the amplified fragment and has an altered stop codon so that translation continues in-frame through the vector c-myc epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *C. aurantiacus* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase (Roche) in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 2.25 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 1 minute, and 72°C for 2.25 minutes; and a final extension for 7 minutes at 72°C. The amplification product was then separated by gel electrophoresis using a 1% TAE-agarose gel. A 0.8 Kb fragment was excised from the gel and purified for each primer pair. The purified fragments were digested with *Kpn* I or *Sal* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Apa* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

50-60 ng of the digested PCR product containing the nucleic acid encoding the *C. aurantiacus* polypeptide having hydratase activity (OS19) and 50 ng of the prepared pESC-Trp vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LB plates containing 100 μ g/mL of carbenicillin (LBC). Individual colonies were screened using colony PCR with the appropriate PCR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells was used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the nucleic acid from genomic DNA.

Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR. A construct with a confirmed sequence was transformed into *S. cerevisiae* strain YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). Transformation reactions were plated on SC-Trp media (see Stratagene pESC Vector Instruction Manual for media recipes). Individual yeast colonies were screened for the presence of the OS19 nucleic acid by colony PCR. Colonies were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 5 units of zymolase and heated at 37°C for 10 minutes. Three µL of this suspension was then used in a 25 µL PCR reaction using the PCR reaction mixture and program described for the colony screen of the *E. coli* transformants. The pESC-Trp vector was also transformed into YPH500 for use as a hydratase assay control and transformants were screened by PCR using GAL1 and GAL10 primers.

B. Construction of the pESC-Trp/OS19/E1 hydratase vector

Plasmid DNA of a pESC-Trp/OS19 construct (*Apa* I-*Sal* I sites) with confirmed sequence and positive assay results was used for insertion of the nucleic acid for the *M. elsdenii* E1 activator polypeptide downstream of the GAL10 promoter. Three µg of plasmid DNA was digested with the restriction enzyme *Cla* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Not* I, and the digest was inactivated by heating to 65°C for 20 minutes. The double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche), separated on a 1% TAE-agarose gel, and gel purified.

The nucleic acid encoding the *M. elsdenii* E1 activator polypeptide was amplified from genomic DNA using the PCR primer pair EINOTF and EICLAR. EINOTF was designed to introduce a *Not* I restriction site and a translation initiation site at the beginning of the amplified fragment. The EICLAR primer was designed to introduce a *Cla* I restriction site at the end of the amplified fragment and to contain an altered translational stop codon to allow in-frame translation of the FLAG epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii* genomic DNA, 0.2 µM of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 µL. The PCR reaction was performed in an MJ Research PTC100

under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 3 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 3 minutes; and a final extension for 7 minutes at 72°C. The amplification product was then separated by gel electrophoresis using a 1% TAE-agarose gel, and a 0.8 Kb fragment was excised and purified. The purified fragment was digested with *Cla* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Not* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

60 ng of the digested PCR product containing the nucleic acid for the *M. elsdenii* E1 activator polypeptide and 70 ng of the prepared pESC-Trp/OS19 hydratase vector were ligated using T4 DNA ligase at 16°C for 16 hours. One µL of the ligation reaction was used to electroporate 40 µL of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC media. Individual colonies were screened using colony PCR with the EINOTF and EICLAR primers. Individual colonies were suspended in about 25 µL of 10 mM Tris, and 2 µL of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 µL of the heated cells used in a 25 µL PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 µM each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA. Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR.

C. Construction of the pESC-Leu/EIIα/EIIβ vector

Three µg of DNA of the vector pESC-Leu was digested with the restriction enzyme *Apa* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Sal* I, and the digest was inactivated by heating to 65°C for 20 minutes. The double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche), separated on a 1% TAE-agarose gel, and gel purified.

The nucleic acid encoding the *M. elsdenii* E2 α polypeptide was amplified from genomic DNA using the PCR primer pair EII α APAF and EII α SALR. EII α APAF was designed to introduce an *Apa* I restriction site and a translation initiation site at the beginning of the amplified fragment. The EII α SALR primer was designed to introduce a

5 *Sal* I restriction site at the end of the amplified fragment and to contain an altered translational stop codon to allow in-frame translation of the c-myc epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100

10 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 3 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 1 minute, and 72°C for 3 minutes; and a final extension for 7 minutes at 72°C. The amplification product was then separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.3 Kb fragment was excised and purified. The purified fragment

15 was digested with *Apa* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Sal* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

80 ng of the digested PCR product containing the nucleic acid encoding the *M. elsdenii* E2 α polypeptide and 80 ng of the prepared pESC-Leu vector were ligated using

20 T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC media. Individual colonies were screened using colony PCR with the EII α APAF and EII α SALR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant

25 suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA. Plasmid DNA was isolated from cultures

30 of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR.

Plasmid DNA of a pESC-Leu/EII α vector with confirmed sequence was used for insertion of the nucleic acid encoding the *M. elsdenii* E2 β polypeptide. Three μ g of plasmid DNA was digested with the restriction enzyme *Spe* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the
5 restriction enzyme *Not* I and gel purified from a 1% TAE-agarose gel. The double-digested vector DNA was then dephosphorylated with shrimp alkaline phosphatase (Roche) and purified with a QIAquick PCR Purification Column.

The nucleic acid encoding the *M. elsdenii* E2 β polypeptide was amplified from genomic DNA using the PCR primer pair EII β NOTF and EII β SPER. The EII β NOTF
10 primer was designed to introduce a *Not* I restriction site and a translation initiation site at the beginning of the amplified fragment. The EII β SPER primer was designed to introduce an *Spe* I restriction site at the end of the amplified fragment and to contain an altered translational stop codon to allow for in-frame translation of the FLAG epitope. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii*
15 genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 3 minutes; 24 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 3 minutes; and a final
20 extension for 7 minutes at 72°C. The amplification product was separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.1 Kb fragment was excised and purified. The purified fragment was digested with *Spe* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Not* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

25 38 ng of the digested PCR product containing the nucleic acid encoding the *M. elsdenii* E2 β polypeptide and 50 ng of the prepared pESC-Leu/EII α vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC plates. Individual colonies were screened using colony PCR with
30 the EII β NOTF and EII β SPER primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant

suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 µL of the heated cells was used in a 25 µL PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 µM each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA.

Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR. A pESC-Leu/EIIα/EIIβ construct with a confirmed sequence was co-transformed along with the pESC-Trp/OS19/EI vector into *S. cerevisiae* strain YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). Transformation reactions were plated on SC-Trp-Leu media. Individual yeast colonies were screened for the presence of the OS19, E1, E2α, and E2β nucleic acid by colony PCR. Colonies were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 5 units of zymolase and heated at 37°C for 10 minutes. Three µL of this suspension was then used in a 25 µL PCR reaction using the PCR reaction mixtures and programs described for the colony screens of the *E. coli* transformants. The pESC-Trp/OS19 and pESC-Leu vectors were also co-transformed into YPH500 for use as a lactyl-CoA dehydratase assay control. These transformants were colony screened using the GAL1 and GAL10 primers (Instruction manual, pESC Yeast Epitope Tagging Vectors, Stratagene).

D. Construction of the pESC-His/D-LDH/PCT vector

Three µg of DNA of the vector pESC-His was digested with the restriction enzyme *Xho* I, and the digest was purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Apa* I and gel purified from a 1% TAE-agarose gel. The double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche) and purified using a QIAquick PCR Purification Column.

The *E. coli* D-LDH gene was amplified from genomic DNA of strain DH10B using the PCR primer pair LDHAPAF and LDHXXHOR. LDHAPAF was designed to introduce an *Apa* I restriction site and a translation initiation site at the beginning of the amplified fragment. The LDHXXHOR primer was designed to introduce an *Xho* I

restriction site at the end of the amplified fragment and to contain the translational stop codon for the D-LDH gene. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *E. coli* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP, and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction
5 was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 2 minutes; 24 cycles of 94°C for 30 seconds, 64°C for 45 seconds, and 72°C for 2 minutes; and a final extension for 7 minutes at 72°C. The amplification product was separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.0 Kb fragment was
10 excised and purified. The purified fragment was digested with *Apa* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Xho* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

80 ng of the digested PCR product containing the *E. coli* D-LDH gene and 80 ng
15 of the prepared pESC-His vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC media. Individual colonies were screened using colony PCR with the LDHAPAF and LDHXHR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the
20 suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from
25 genomic DNA. Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR.

Plasmid DNA of a pESC-His/D-LDH construct with a confirmed sequence was used for insertion of the nucleic acid encoding the *M. elsdenii* PCT polypeptide. Three μ g of plasmid DNA was digested with the restriction enzyme *Pac* I, and the digest was
30 purified using a QIAquick PCR Purification Column. The vector DNA was then digested with the restriction enzyme *Spe* I and gel purified from a 1% TAE-agarose gel. The

double-digested vector DNA was dephosphorylated with shrimp alkaline phosphatase (Roche) and purified with a QIAquick PCR Purification Column.

The nucleic acid encoding the *M. elsdenii* PCT polypeptide was amplified from genomic DNA using the PCR primer pair PCTSPEF and PCTPACR. PCTSPEF was
5 designed to introduce an *Spe* I restriction site and a translation initiation site at the beginning of the amplified fragment. The PCTPACR primer was designed to introduce a *Pac* I restriction site at the end of the amplified fragment and to contain the translational stop codon for the PCT gene. The PCR mix contained the following: 1X Expand PCR buffer, 100 ng *M. elsdenii* genomic DNA, 0.2 μ M of each primer, 0.2 mM each dNTP,
10 and 5.25 units of Expand DNA Polymerase in a final volume of 100 μ L. The PCR reaction was performed in an MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 1 minute; 8 cycles of 94°C for 30 seconds, 56°C for 45 seconds, and 72°C for 2.5 minutes; 24 cycles of 94°C for 30 seconds, 64°C for 45 seconds, and 72°C for 2.5 minutes; and a final extension for 7 minutes at 72°C. The
15 amplification product was separated by gel electrophoresis using a 1% TAE-agarose gel, and a 1.55 Kb fragment was excised and purified. The purified fragment was digested with *Pac* I restriction enzyme, purified with a QIAquick PCR Purification Column, digested with *Spe* I restriction enzyme, purified again with a QIAquick PCR Purification Column, and quantified on a minigel.

20 95 ng of the digested PCR product containing the nucleic acid encoding the *M. elsdenii* PCT polypeptide and 75 ng of the prepared pESC-His/D-LDH vector were ligated using T4 DNA ligase at 16°C for 16 hours. One μ L of the ligation reaction was used to electroporate 40 μ L of *E. coli* Electromax™ DH10B™ cells. The electroporated cells were plated onto LBC plates. Individual colonies were screened using colony PCR
25 with the PCTSPEF and PCTPACR primers. Individual colonies were suspended in about 25 μ L of 10 mM Tris, and 2 μ L of the suspension was plated on LBC media. The remnant suspension was heated for 10 minutes at 95°C to break open the bacterial cells, and 2 μ L of the heated cells used in a 25 μ L PCR reaction. The PCR mix contained the following: 1X Taq buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq
30 DNA polymerase per reaction. The PCR program used was the same as described above for amplification of the gene from genomic DNA.

Plasmid DNA was isolated from cultures of colonies having the desired insert and was sequenced to confirm the lack of nucleotide errors from PCR. A construct with a confirmed sequence was transformed into *S. cerevisiae* strain YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). Transformation reactions were plated on SC-His media. Individual yeast colonies were screened for the presence of the D-LDH and PCT genes by colony PCR. Colonies were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 5 units of zymolase and heated at 37°C for 10 minutes. Three µL of this suspension was then used in a 25 µL PCR reaction using the PCR reaction mixture and program described for the colony screen of the *E. coli* transformants. The pESC-His vector was also transformed into YPH500 for use as an assay control, and transformants were screened by PCR using GAL1 and GAL10 primers.

Example 13 - Expression of Enzymes in *S. cerevisiae*

A. Hydratase Activity in Transformed Yeast

Individual colonies carrying the pESC-Trp/OS19 construct or the pESC-Trp vector (negative control) were used to inoculate 5 mL cultures of SC-Trp media containing 2% glucose. These cultures were grown for 16 hours at 30°C and used to inoculate 35 mL of the same media. The subcultures were grown for 7 hours at 30°C, and their OD_{600s} were determined. A volume of cells giving an OD x volume equal to 40 was pelleted, washed with SC-Trp media with no carbon source, and repelleted. The cells were suspended in 5 mL of SC-Trp media containing 2% galactose and used to inoculate a total volume of 100 mL of this media. Cultures were grown for 17.5 hours at 30°C and 250 rpm. Cells were then pelleted, rinsed in 0.85% NaCl, and repelleted. Cell pellets (70 mg) were suspended in 140 µL of 50 mM TrisHCl, pH 7.5, and an equal volume (pellet plus buffer) of pre-rinsed glass beads (Sigma, 150-212 microns) was added. This mixture was vortexed for 30 seconds and placed on ice for 1 minute, and the vortexing/cooling cycle was repeated 8 additional times. The cells were then centrifuged for 6 minutes at 5,000g, and the supernatant was removed to a fresh tube. The beads/pellet were washed twice with 250 µL of buffer, centrifuged, and the supernatants joined with the first supernatant.

An *E. coli* strain carrying the pETBlue-1/OS19 construct, described previously, was used as a positive control for hydratase assays. A culture of this strain was grown to saturation overnight and diluted 1:20 the following morning in fresh LBC media. The culture was grown at 37°C and 250 rpm to an OD₆₀₀ of 0.6, at which point it was induced with IPTG at a final concentration of 1 mM. The culture was incubated for an additional two hours at 37°C and 250 rpm. Cells were pelleted, washed with 0.85 % NaCl, and repelleted. Cells were disrupted using BugBuster™ Protein Extraction Reagent and Benzonase® (Novagen) as per manufacturer's instructions with a 20 minute incubation at room temperature. After centrifugation at 16,000g and 4°C, the supernatant was transferred to a new tube and used in the activity assay.

Total protein content of cell extracts from *S. cerevisiae* described above were quantified using a microplate Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The OS19 constructs (both *Apa* I-*Sal* I and *Apa* I-*Kpn* I-sites) in YPH500, the pESC-Trp negative control in YPH500, and the pETBlue-1/OS19 construct in *E. coli* were tested for their ability to convert acrylyl-CoA to 3-hydroxypropionyl-CoA. The assay was conducted as previously described for the pETBlue-1/OS19 constructs in the *E. coli* Tuner strain. When cell extract of the negative control strain was added to the reaction mixture containing acrylyl-CoA, one dominant peak of MW 823 was exhibited. This peak corresponds to acrylyl-CoA and indicates that acrylyl-CoA was not converted to any other product. When cell extract of the strain carrying a pESC-Trp/OS19 construct (either *Apa* I-*Sal* I or *Apa* I-*Kpn* I-sites) was added to the reaction mix, the dominant peak shifted to MW 841, which corresponds to 3-hydroxypropionyl-CoA. The reaction mix from the *E. coli* control also showed the MW 841 peak. A time course study was conducted for the pESC-Trp/OS19(*Apa* I-*Sal* I) construct, which measured the appearance of the MW 841 and MW 823 peaks after 0, 1, 3, 7, 15, 30, and 60 minutes of reaction time. An increase in the 3-hydroxypropionyl-CoA peak was seen over time with the cell extracts from both this construct and the *E. coli* control, whereas cell extract from the YPH500 strain with vector only showed a dominant acrylyl-CoA peak.

B. Propionyl CoA-Transferase Activity in Transformed Yeast

Individual colonies of *S. cerevisiae* strain YPH500 carrying the pESC-His/D-LDH or pESC-His/D-LDH/PCT construct or the pESC-His vector with no insert (negative control) were used to inoculate 5 mL cultures of SC-His media containing 2% glucose.

- 5 These cultures were grown for 16 hours at 30°C and 250 rpm and were then used to inoculate 35 mL of the same media. The subcultures were grown for 7 hours at 30°C, and their OD_{600s} were determined. For each strain, a volume of cells giving an OD x volume equal to 40 was pelleted, washed with SC-His media with no carbon source, and repelleted. The cells were suspended in 5 mL of SC-His media containing 2% galactose
- 10 and used to inoculate a total volume of 100 mL of this media. Cultures were grown for 16.75 hours at 30°C and 250 rpm. Cells were then pelleted, rinsed in 0.85% NaCl, and repelleted. Cell pellets (70 mg) were suspended in 140 µL of 100 mM potassium phosphate buffer, pH 7.5, and an equal volume (pellet plus buffer) of pre-rinsed glass beads (Sigma, 150-212 microns) was added. This mixture was vortexed for 30 seconds
- 15 and placed on ice for 1 minute, and the vortexing/cooling cycle was repeated 8 additional times. The cells were then centrifuged for 6 minutes at 5,000g, and the supernatant was removed to a fresh tube. The beads/pellet were washed twice with 250 µL of buffer and centrifuged, and the supernatants joined with the first supernatant.

- 20 An *E. coli* strain carrying the pETBlue-1/PCT construct, described previously, was used as a positive control for propionyl CoA transferase assays. A culture of this strain was grown to saturation overnight and diluted 1:20 the following morning in fresh LB media containing 100 µg/mL of carbenicillin. The culture was grown at 37°C and 250 rpm to an OD₆₀₀ of 0.6, at which point it was induced with IPTG at a final concentration of 1 mM. The culture was incubated for an additional two hours at 37°C
- 25 and 250 rpm. Cells were pelleted, washed with 0.85 % NaCl, and repelleted. Cells were disrupted using BugBuster™ Protein Extraction Reagent and Benzonase® (Novagen) as per manufacturer's instructions with a 20 minute incubation at room temperature. After centrifugation at 16,000g and 4°C, the supernatant was transferred to a new tube and used in the activity assay.

- 30 Total protein content of cell extracts was quantified using a microplate Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The D-LDH and D-LDH/PCT constructs in *S.*

cerevisiae strain YPH500, the pESC-His negative control in YPH500, and the pETBlue-1/PCT construct in *E. coli* were tested for their ability to catalyze the conversion of propionyl-CoA and acetate to acetyl-CoA and propionate. The assay mixture used was that previously described for the pETBlue-1/PCT constructs in the *E. coli* Tuner strain.

5 When 1 μ g of total cell extract protein of the negative control strain or the YPH500/pESC-His/D-LDH strain was added to the reaction mixture, no increase in absorbance (0.00 to 0.00) was seen over 11 minutes. Increases in absorbance from 0.00 to 0.04 and from 0.00 to 0.06 were seen, respectively, with 1 μ g of cell extract protein from the YPH500/pESC-His/D-LDH/PCT strain and the *E. coli*/PCT strain. With 2 mg
10 of total cell extract protein, the negative control strain and the YPH500/pESC-His/D-LDH strain showed an increase in absorbance from 0.00 to 0.01 over 11 minutes, whereas increases from 0.00 to 0.10 and 0.00 to 0.08 were seen, respectively, with the YPH500/pESC-His/D-LDH/PCT strain and the *E. coli*/PCT strain.

15 C. Lactyl-CoA Dehydratase Activity in Transformed Yeast

Individual colonies of *S. cerevisiae* strain YPH500 carrying the pESC-His/D-LDH or pESC-His/D-LDH/PCT construct or the pESC-His vector with no insert (negative control) were used to inoculate 5 mL cultures of SC-His media containing 4% glucose. These cultures were grown for 23 hours at 30°C and used to inoculate 35 mL of SC-His
20 media containing 2 % raffinose. The subcultures were grown for 8 hours at 30°C, and their OD_{600s} were determined. For each strain, a volume of cells giving an OD x volume equal to 40 was pelleted, resuspended in 10 mL of SC-His media containing 2% galactose, and used to inoculate a total volume of 100 mL of this media. Cultures were grown for 17 hours at 30°C and 250 rpm. Cells were then pelleted, rinsed in 0.85% NaCl,
25 and repelleted. Cell pellets (190 mg) were suspended in 380 μ L of 100 mM potassium phosphate buffer, pH 7.5, and an equal volume (pellet plus buffer) of pre-rinsed glass beads (Sigma, 150-212 microns) was added. This mixture was vortexed for 30 seconds and placed on ice for 1 minute, and the vortexing/cooling cycle was repeated 7 additional times. The cells were then centrifuged for 6 minutes at 5,000 g and the supernatant was
30 removed to a fresh tube. The beads/pellet were washed twice with 300 μ L of buffer and centrifuged, and the supernatants joined with the first supernatant.

An anaerobically-grown culture of *E. coli* strain DH10B was used as a positive control for D-LDH assays. A culture of this strain was grown to saturation overnight and diluted 1:20 the following morning in fresh LB media. The culture was grown anaerobically at 37°C for 7.5 hours. Cells were pelleted, washed with 0.85 % NaCl, and repelleted. Cells were disrupted using BugBuster™ Protein Extraction Reagent and Benzonase® (Novagen) as per manufacturer's instructions with a 20-minute incubation at room temperature. After centrifugation at 16,000g and 4°C, the supernatant was transferred to a new tube and used in the activity assay.

Total protein content of cell extracts was quantified using a microplate Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The D-LDH and D-LDH/PCT constructs in YPH500, the pESC-His negative control in YPH500, and the anaerobically-grown *E. coli* strain were tested for their ability to catalyze the conversion of pyruvate to lactate by assaying the concurrent oxidation of NADH to NAD. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.5, 0.2 mM NADH, and 0.5-1.0 µg of cell extract. The reaction was started by the addition of sodium pyruvate to a final concentration of 5 mM, and the decrease in absorbance at 340 nm was measured over 10 minutes. When 0.5 µg of total cell extract protein of the negative control strain was added to the reaction mixture, a decrease in absorbance from -0.01 to -0.02 was seen over 200 seconds. A decrease in absorbance from -0.21 to -0.47 and -0.20 to -0.47 over 200 seconds was seen, respectively, for cell extract from the YPH500/pESC-His/D-LDH or YPH500/pESC-His/D-LDH/PCT strains. 0.5 µL (7.85 µg) of cell extract from the anaerobically-grown *E. coli* strain showed a decrease in absorbance very similar to that for 1 µg of cell extract of the YPH500/pESC-His/D-LDH/PCT strain. When 4 µg of cell extract was used, the YPH500/pESC-His/D-LDH/PCT strain showed a decrease in absorbance from -0.18 to -0.60 over 10 minutes, whereas the negative control strain showed no decrease in absorbance (-0.08 to -0.08).

D. Demonstration of 3-HP production in *S. cerevisiae*

The pESC-Trp/OS19/EI, pESC-Leu/EIIa/EIIB, and pESC-His/D-LDH/PCT constructs were transformed into a single strain of *S. cerevisiae* YPH500 using a Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA). A negative control

strain was also developed by transformation of the pESC-Trp, pESC-Leu, and pESC-His vectors into a single YPH500 strain. Transformation reactions were plated on SC-Trp-Leu-His media. Individual yeast colonies were screened by colony PCR for the presence or absence of nucleic acid corresponding to each construct.

5 The strain carrying all six genes and the negative control strain were grown in 5 mL of SC-Trp-Leu-His media containing 2% glucose. These cultures were grown for 31 hours at 30°C, and 2 mL was used to inoculate 50 mL of the same media. The subcultures were grown for 19 hours at 30°C, and their OD600s were determined. For each strain, a volume of cells giving an OD x volume equal to 100 was pelleted, washed
10 with SC-Trp-Leu-His media with no carbon source, and repelleted. The cells were suspended in 10 mL of SC-Trp-Leu-His media containing 2% galactose and 2% raffinose and used to inoculate a total volume of 250 mL of this media. The cultures were grown in bottles at 30°C with no shaking, and samples were taken at 0, 4.5, 20, 28.5, 45, and 70 hours. Samples were spun down to remove cells and the supernatant was filtered using
15 0.45 micron Acrodisc Syringe Filters (Pall Gelman Laboratory, Ann Arbor, MI).

100 microliters of the filtered broth was used to derive CoA esters of any lactate or 3-HP in the broth using 6 micrograms of purified propionyl-CoA transferase, 50 mM potassium phosphate buffer (pH 7.0), and 1 mM acetyl-CoA. The reaction was allowed to proceed at room temperature for 15 minutes and was stopped by adding 1 volume 10%
20 trifluoroacetic acid. The reaction mixtures were purified using Sep Pak C18 columns as previously described and analyzed by LC/MS.

Example 14 Constructing a Biosynthetic Pathway that Produces Organic Acids from β -alanine

25 One possible pathway to 3-HP from β -alanine involves the use of a polypeptide having CoA transferase activity (e.g., an enzyme from a class of enzymes that transfers a CoA group from one metabolite to the other). As shown in Figure 54, β -alanine can be converted to β -alanyl-CoA using a polypeptide having CoA transferase activity and CoA donors such as acetyl-CoA or propionyl-CoA. Alternatively, β -alanyl-CoA can be
30 generated by the action of a polypeptide having CoA synthetase activity. The β -alanyl-CoA can be deaminated to form acrylyl-CoA by a polypeptide having β -alanyl-CoA

ammonia lyase activity. The hydration of acrylyl-CoA at the β position to yield 3-HP-CoA can be carried out by a polypeptide having 3-HP-CoA dehydratase activity. The 3-HP-CoA can act as a CoA donor for β -alanine, a reaction that can be catalyzed a polypeptide having CoA transferase activity, thus yielding 3-HP as a product.

- 5 Alternatively, 3-HP-CoA can be hydrolyzed to yield 3-HP by a polypeptide having specific CoA hydrolase activity.

Methods for isolating, sequencing, expressing, and testing the activity of a polypeptide having CoA transferase activity are described herein.

10 A. Isolation of a polypeptide having β -alanyl-CoA Ammonia Lyase Activity

Polypeptides having β -alanyl-CoA ammonia lyase activity can catalyze the conversion of β -alanyl-CoA into acrylyl-CoA. The activity of such polypeptides has been described by Vagelos *et al.* (*J. Biol. Chem.*, 234:490-497 (1959)) in *Clostridium propionicum*. This polypeptide can be used as part of the acrylate pathway in *Clostridium*

15 *propionicum* to produce propionic acid.

- C. propionicum* was grown at 37°C in an anoxic medium containing 0.2% yeast extract, 0.2% trypticase peptone, 0.05% cysteine, 0.5% β -alanine, 0.4% VRB-salts, 5 mM potassium phosphate, pH 7.0. The cells were harvested after 12 hours and washed twice with 50 mM potassium phosphate (Kpi), pH 7.0. About 2 g of wet packed cells were re-
- 20 suspended in 40 mL of Kpi, pH 7.0, 1mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT (Buffer A), and homogenized by sonication at about 85-100 W power using a 3mm tip (Branson sonifier 250). Cell debris was removed by centrifugation at 100,000g for 45 minutes in a Centricon T-1080 Ultra centrifuge, and the cell free extract (~ 110 U/mg activity) was subjected to anion exchange chromatography on Source-15Q-material. The Source-15Q
- 25 column was loaded with 32 mL of cell free extract. The column was developed by a linear gradient of 0 M to 0.5 M NaCl within 10 column volumes. The polypeptide eluted between 70-110 mM NaCl.

- The solution was adjusted to a final concentration of 1 M $(NH_4)_2SO_4$ and applied onto a Resource-Phe column equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. The
- 30 polypeptide did not bind to this column.

The final preparation was obtained after concentration in an Amicon chamber (filter cut-off 30 kDa). The functional polypeptide is composed of four polypeptide sub-units, each having a molecular mass of 16 kDa. The polypeptide had a final specific activity of 1033 U/mg in the standard assay (see below).

5 The polypeptide sample after every purification step was separated on a 15% SDS-PAGE gel. The gel was stained with 0.1% Coomassie R 250, and the destaining was achieved by using 7.1% acetic acid/5% ethanol solution.

 The polypeptide was desalted by RP-HPLC and subjected to N-terminal sequencing by gas phase Edman degradation. The results of this analysis yielded a 35
10 amino acid N-terminal sequence of the polypeptide. The sequence was as follows: MV-GKKVVHHLMMMSAKDAHYTGNLVNGARIVNQWGD (SEQ ID NO:177).

B. Amplification of a Gene Fragment

 The 35 amino acid sequence of the polypeptide having β -alanine-CoA ammonia
15 lyase activity was used to design primers with which to amplify the corresponding DNA from genome of *C. propionicum*. Genomic DNA from *C. propionicum* was isolated using the Gentra Genomic DNA isolation Kit (Gentra Systems, Minneapolis) following the genomic DNA protocol for gram-positive bacteria. A codon usage table for
20 *Clostridium propionicum* was used to back translate the seven amino acids on either end of the amino acid sequence to obtain 20-nucleotide degenerate primers:

ACLF: 5'-ATGGTWGGYAARAARGTWGT-3' (SEQ ID NO:178)

ACLR: 5'-TCRCCCCAYTGRTTWACRAT-3' (SEQ ID NO:179)

 The primers were used in a 50 μ L PCR reaction containing 1X Taq PCR buffer, 0.6 μ M each primer, 0.2 mM each dNTP, 2 units of Taq DNA polymerase (Roche
25 Molecular Biochemicals, Indianapolis, IN), 2.5% (v/v) DMSO, and 100 ng of genomic DNA. PCR was conducted using a touchdown PCR program with 4 cycles at an annealing temperature of 58°C, 4 cycles at 56°C, 4 cycles at 54°C, and 24 cycles at 52°C. Each cycle used an initial 30 second denaturing step at 94°C and a 1.25 minute extension
30 at 72°C, and the program had an initial denaturation step at 94°C for 2 minutes and final extension at 72°C for 5 minutes. The amounts of PCR primer used in the reaction were increased three-fold above typical PCR amounts due to the amount of degeneracy in the

3' end of the primer. In addition, separate PCR reactions containing each individual primer were made to identify PCR product resulting from single degenerate primers. Twenty μ L of each PCR product was separated on a 2.0% TAE (Tris-acetate-EDTA)-agarose gel.

5 A band of about 100 bp was produced by the reaction containing both the forward and reverse primers, but was not present in the individual forward and reverse primer control reactions. This fragment was excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Four microliters of the purified band was ligated into pCRII-TOPO vector and transformed by a heat-shock method into TOP10 *E. coli*
10 cells using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 50 μ g/mL of kanamycin and 50 μ g/mL of 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside (X-gal). Individual, white colonies were resuspended in 25 μ L of 10 mM Tris and heated for 10 minutes at 95°C to break open the bacterial cells. Two microliters of the heated cells were used in a 25 μ L PCR reaction.
15 using M13R and M13F universal primers homologous to the pCRII-TOPO vector. The PCR mix contained the following: 1X Taq PCR buffer, 0.2 μ M each primer, 0.2 mM each dNTP, and 1 unit of Taq DNA polymerase per reaction. The PCR reaction was performed in a MJ Research PTC100 under the following conditions: an initial denaturation at 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 52°C for 1 minute,
20 and 72°C for 1.25 minutes; and a final extension for 7 minutes at 72°C. Plasmid DNA was obtained (QIAprep Spin Miniprep Kit, Qiagen) from cultures of colonies showing the desired insert and was used for DNA sequencing with M13R universal primer. The following nucleic acid sequence was internal to the degenerate primers and corresponds to a portion of the 35 amino acid residue sequence: 5'-ACATCATTTAATGATGA-
25 GCGCAAAAGATGCTCACTATACTGGAACTTAGTAAACGGCGCTAGA-3' (SEQ ID NO:180).

C. Genome Walking to Obtain the Complete Coding Sequence

Primers for conducting genome walking in both upstream and downstream
30 directions were designed using the portion of the nucleic acid sequence that was internal to the degenerate primers. The primer sequences were as follows:

ACLGSP1F: 5'-GTACATCATTTAATGATGAGCGCAAAAGATG-3' (SEQ ID NO:181)

ACLGSP2F: 5'-GATGCTCACTATACTGGAACTTAGTAAAC-3' (SEQ ID NO:182)

5 ACLGSP1R: 5'-ATTCTAGCGCCGTTTACTAAGTTTCCAG-3' (SEQ ID NO:183)

ACLGSP2R: 5'-CCAGTATAGTGAGCATCTTTTGGGCTCATC-3' (SEQ ID NO:184)

GSP1F and GSP2F are primers facing downstream, GSP1R and GSP2R are primers facing upstream, and GSP2F and GSP2R are primers nested inside GSP1F and GSP1R, respectively. Genome walking libraries were constructed according to the manual for CLONTECH's Universal Genome Walking Kit (CLONTECH Laboratories, Palo Alto, CA), with the exception that the restriction enzymes *Ssp* I and *Hinc* II were used in addition to *Dra* I, *EcoR* V, and *Pvu* II. PCR was conducted in a Perkin Elmer 9700 Thermocycler using the following reaction mix: 1X XL Buffer II, 0.2 mM each dNTP, 1.25 mM Mg(OAc)₂, 0.2 μM each primer, 2 units of rTth DNA polymerase XL (Applied Biosystems, Foster City, CA), and 1 μL of library per 50 μL reaction. First round PCR used an initial denaturation at 94°C for 5 seconds; 7 cycles consisting of 2 sec at 94°C and 3 min at 70°C; 32 cycles consisting of 2 sec at 94°C and 3 min at 64°C; and a final extension at 64°C for 4 min. Second round PCR used an initial denaturation at 94°C for 15 seconds; 5 cycles consisting of 5 sec at 94°C and 3 min at 70°C; 26 cycles consisting of 5 sec at 94°C and 3 min at 64°C; and a final extension at 66°C for 7 min. Twenty μL of each first and second round product was run on a 1.0% TAE-agarose gel. In the second round PCR for the forward reactions, a 1.4 Kb band was obtained for *Dra* I, a 1.5 Kb band for *Hinc* II, a 4.0 Kb band for *Pvu* II, and 2.0 and 2.6 Kb bands were obtained for *Ssp* I. In the second round PCR for the reverse reactions, a 1.5 Kb band was obtained for *Dra* I, a 0.8 Kb band for *EcoR* V, a 2.0 Kb band for *Hinc* II, a 2.9 Kb band for *Pvu* II, and a 1.5 Kb band was obtained for *Ssp* I. Several of these fragments were gel purified, cloned, and sequenced.

The coding sequence of the polypeptide having β-alanyl-CoA ammonia lyase activity is set forth in SEQ ID NO:162. This coding sequence encodes the amino acid sequence set forth in SEQ ID NO:160. The coding sequence was cloned and expressed in

bacterial cells. A polypeptide with the expected size was isolated and tested for enzymatic activity.

The isolation of a nucleic acid molecule encoding a polypeptide having 3-HP-CoA dehydratase activity (e.g., the seventh enzymatic activity in Figure 54, which can be accomplished with a polypeptide having the amino acid sequence set forth in SEQ ID NO:41) is described herein. This polypeptide in combination with a polypeptide having CoA transferase activity (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO:2) and a polypeptide having β -alanyl-CoA ammonia lyase activity (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO: 160) provides one method of making 3-HP from β -alanine.

Example 15 Constructing a Biosynthetic Pathway that Produces Organic Acids from β -alanine

In another pathway, β -alanine generated from aspartate can be deaminated by a polypeptide having 4, 4-aminobutyrate aminotransferase activity (Figure 55). This reaction also can regenerate glutamate that is consumed in the formation of aspartate. The deamination of β -alanine can yield malonate semialdehyde, which can be further reduced to 3-HP by a polypeptide having 3-hydroxypropionate dehydrogenase activity or a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity. Such polypeptides can be obtained as follows.

A. Cloning *gabT* (4-aminobutyrate aminotransferase) from *C. acetobutylicum*

The following PCR primers were designed based on a published sequence for a *gabT* gene from *Clostridium acetobutylicum* (GenBank# AE007654):

Cac aba nco sen: 5'-GAGCCATGGAAGAAATAAATGCTAAAG- 3' (SEQ ID NO:185)
Cac aba bam anti: 5'-AGAGGATGGCTTTTAAATCGCTATTC- 3' (SEQ ID NO:186)

The primers introduced a *NcoI* site at the 5' end and a *BamHI* site at the 3' end. A PCR reaction was set up using chromosomal DNA from *C. acetobutylicum* as the template.

	H ₂ O	80.75 μ L	PCR Program
	Taq Plus Long 10x Buffer	10 μ L	94° C 5 minutes
	dNTP mix (10 mM)	3 μ L	25 cycles of:
	Cac aba nco sen (20 mM)	2 μ L	94° C 30 seconds
5	Cac aba bam anti (20 mM)	2 μ L	50° C 30 seconds
	<i>C. acetobutylicum</i> DNA (~100 ng)	1 μ L	72° C 80 seconds + 2
	Taq Plus Long (5 U/mL)	1 μ L	seconds/cycle
	Pfu (2.5 U/mL)	0.25 μ L	1 cycle of:
			68° C 7 minutes
10			4° C until use

Upon agarose gel analysis a single band was observed of ~1.3 Kb in size. This fragment was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into pCRII TOPO using the TOPO Zero Blunt PCR cloning kit (Invitrogen, Carlsbad, CA). 1 μ L of the pCRII TOPO ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 μ g/mL) plates. Single colonies of the transformant grown overnight in LB/kanamycin media, and the plasmid DNA was extracted using a Mini prep kit (Qiagen, Valencia, CA). The super-coiled plasmid DNA was separated on a 1% agarose gel digested, and the colonies with insert were selected. The insert was sequenced to confirm the sequence and its quality.

The plasmid having the correct insert was digested with restriction enzyme *Nco* I and *Bam*HI. The digested insert was gel isolated and ligated to pET28b expression vector that was also restricted with *Nco* I and *Bam*HI enzymes. 1 μ L of ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were recovered for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 μ g/mL) plates. The super-coiled plasmid DNA was separated on a 1% agarose gel digested, and the colonies with insert were selected. The plasmid with the insert was isolated using a Mini prep kit (Qiagen, Valencia, CA), and 1 μ L of this plasmid DNA was used to transform electrocompetent BL21(DE3) (Novagen, Madison, WI). These cells were used to check the expression of a polypeptide having 4-aminobutyrate aminotransferase activity.

B. Cloning mmsB (3-hydroxyisobutyrate dehydrogenase) from *P. aeruginosa*

The following PCR primers was designed based on a published sequence for a mmsB gene from *Pseudomona aeruginosa* (GenBank# M84911):

Ppu hid nde sen: 5'-ATACATATGACCGACCGACATCGCATT-3' (SEQ ID NO:186)

5 Ppu hid sal anti: 5'-ATAGTCGACGGGTCAGTCCTTGCCGCG-3' (SEQ ID NO:187)

The primers introduced a *Nde* I site at the 5' end and a *Bam*HI site at the 3' end.

H ₂ O	80.75 µL	PCR Program
Taq Plus Long 10x Buffer	10 µL	94° C 5 minutes
dNTP mix (10 mM)	3 µL	25 cycles of: 94° C 30 seconds 55° C 30 seconds 72° C 90 seconds + 2 seconds/cycle
Ppu hid.nde sen (20 µM)	2 µL	68° C 7 minutes
Ppu hid sal anti (20 µM)	2 µL	4° C until use
<i>C. acetobutylicum</i> DNA (~100 ng)	1 µL	
Taq Plus Long (Stratagene, La Jolla, CA)	1 µL	
Pfu (Stratagene, La Jolla, CA)	0.25 µL	

10 A PCR reaction was set up using chromosomal DNA from *P. aeruginosa* as the template. Chromosomal DNA was obtained from ATCC (Manassas, VA) *P. aeruginosa* 17933D.

15 Upon agarose gel analysis, a single band was observed of ~1.6 Kb in size. This fragment was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into pCRII TOPO using the TOPO Zero Blunt PCR cloning kit (Invitrogen, Carlsbad, CA). 1 µL of the pCRII TOPO ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were recovered for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 µg/mL) plates. Single colonies of the transformant grown overnight in LB/kanamycin media, and the plasmid DNA was extracted using a Mini prep kit (Qiagen, Valencia, CA). The super-coiled plasmid DNA

was separated on a 1% agarose gel and digested, and the colonies with insert were selected. The insert was sequenced to confirm the sequence and its quality.

The plasmid having the correct insert was digested with restriction enzyme *Nde* I and *Bam*HI. The digested insert was gel isolated and ligated to pET30a expression vector that was also restricted with *Nde* I and *Bam*HI enzymes. 1 μ L of ligation mix was used to transform chemically competent TOP10 *E. coli* cells. The cells were recovered for 1 hour in SOC media, and the transformants were selected on LB/kanamycin (50 μ g/mL) plates. The super-coiled plasmid DNA was separated on a 1% agarose gel and digested, and the colonies with insert were selected. The plasmid with the insert was isolated using a Mini prep kit (Qiagen, Valencia, CA), and 1 μ L of this plasmid DNA was used to transform electrocompetent BL21(DE3) (Novagen, Madison, WI). These cells were used to check the expression of a polypeptide having 3-hydroxyisobutyrate dehydrogenase activity.

15

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

20

WHAT IS CLAIMED IS:

1. A cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.
- 5 2. The cell of claim 1, wherein said cell comprises an activity selected from the group consisting of E1 activator activity, E2 α activity, and E2 β activity.
3. The cell of claim 1, wherein said cell comprises 3-hydroxypropionyl-CoA dehydratase activity.
- 10 4. The cell of claim 1, wherein said cell comprises CoA transferase activity.
5. The cell of claim 1, wherein said cell comprises an exogenous nucleic acid comprising:
15 (a) a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; or
(b) a nucleic acid sequence that shares at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162,
20 or 163.
6. The cell of claim 1, wherein said cell comprises 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
- 25 7. The cell of claim 1, wherein said cell comprises lipase activity.
8. The cell of claim 1, wherein said cell produces 3-HP.
9. The cell of claim 1, wherein said cell produces an ester of 3-HP.
- 30

10. The cell of claim 9, wherein said ester is selected from the group consisting of methyl 3-hydroxypropionate, ethyl 3-hydroxypropionate, propyl 3-hydroxypropionate, butyl 3-hydroxypropionate, and 2-ethylhexyl 3-hydroxypropionate.
- 5 11. The cell of claim 1, wherein said cell comprises CoA synthetase activity.
12. The cell of claim 1, wherein said cell comprises poly hydroxyacid synthase activity.
- 10 13. The cell of claim 1, wherein said cell produces polymerized 3-HP.
14. The cell of claim 1, wherein said cell is prokaryotic.
15. The cell of claim 1, wherein said cell is selected from the group consisting of
- 15 yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
16. A cell comprising CoA synthetase activity, lactyl-CoA dehydratase activity, and poly hydroxyacid synthase activity.
- 20 17. The cell of claim 16, wherein said cell comprises an activity selected from the group consisting of E1 activator activity, E2 α activity, and E2 β activity.
18. The cell of claim 16, wherein the cell produces polymerized acrylate.
- 25 19. The cell of claim 16, wherein said cell is prokaryotic.
20. The cell of claim 16, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
- 30 21. A cell comprising CoA transferase activity, lactyl-CoA dehydratase activity, and lipase activity.

22. The cell of claim 21, wherein said cell comprises an activity selected from the group consisting of E1 activator activity, E2 α activity, and E2 β activity.
- 5 23. The cell of claim 21, wherein said cell produces an ester of acrylate.
24. The cell of claim 23, wherein said ester is selected from the group consisting of methyl acrylate, ethyl acrylate, propyl acrylate, and butyl acrylate.
- 10 25. The cell of claim 21, wherein said cell is prokaryotic.
26. The cell of claim 21, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
- 15 27. An polypeptide comprising an amino acid sequence selected from the group consisting of:
- (a) a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161;
 - (b) a sequence having at least 10 contiguous amino acid residues of a sequence set
 - 20 forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161;
 - (c) a sequence that has at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161;
 - (d) a sequence that has at least 65 percent sequence identity with at least 10 contiguous amino acid residues of a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35,
 - 25 37, 39, 41, 141, 160, or 161; and
 - (e) a sequence set forth in SEQ ID NO:2, 10, 18, 26, 35, 37, 39, 41, 141, 160, or 161 that contains at least one conservative substitution.
28. A nucleic acid molecule comprising a nucleic acid sequence that encodes the
- 30 polypeptide of claim 27.

29. A transformed cell comprising at least one exogenous nucleic acid molecule, wherein said molecule comprises a nucleic acid sequence that encodes the polypeptide of claim 27.
- 5 30. The cell of claim 29, wherein the cell produces 3-HP.
31. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes an E2 α polypeptide of an enzyme having lactyl-CoA dehydratase activity.
- 10 32. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes an E2 β polypeptide of an enzyme having said lactyl-CoA dehydratase activity.
33. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes a polypeptide having 3-hydroxypropionyl-CoA dehydratase activity or CoA transferase activity.
- 15 34. The cell of claim 29, wherein said exogenous nucleic acid molecule encodes a polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
- 20 35. The cell of claim 29, wherein the cell comprises lipase activity.
36. The cell of claim 29, wherein the cell produces an ester of 3-HP.
- 25 37. The cell of claim 36, wherein said ester is selected from the group consisting of methyl 3-hydroxypropionate, ethyl 3-hydroxypropionate, propyl 3-hydroxypropionate, butyl 3-hydroxypropionate, and 2-ethylhexyl 3-hydroxypropionate.
38. The cell of claim 29, wherein said cell comprises CoA synthetase activity.
- 30 39. The cell of claim 29, wherein said cell produces polymerized 3-HP.

40. The cell of claim 29, wherein said cell is prokaryotic.
41. The cell of claim 29, wherein said cell is selected from the group consisting of
5 *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
42. The cell of claim 29, wherein the cell is a yeast cell.
43. A specific binding agent that specifically binds to the polypeptide of claim 27.
- 10 44. An isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:
- (a) a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163;
- 15 (b) a sequence having at least 10 contiguous nucleotides of a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163;
- (c) a sequence that has at least 65 percent sequence identity with a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163;
- 20 (d) a sequence that has at least 65 percent sequence identity with at least 10 contiguous nucleotides of a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163; and
- (e) a sequence that hybridize under moderately stringent conditions a sequence set forth in SEQ ID NO:1, 9, 17, 25, 33, 34, 36, 38, 40, 42, 129, 140, 142, 162, or 163.
- 25 45. A production cell comprising an isolated nucleic acid molecule of claim 44 that is exogenous to said production cell.
46. The cell of claim 45, wherein said isolated nucleic acid molecule encodes a polypeptide having an enzymatic activity selected from the group consisting of CoA
30 transferase activity, lactyl-CoA dehydratase activity, CoA synthase activity, CoA

dehydratase activity, dehydrogenase activity, malonyl-CoA reductase activity, and 3-hydroxypropionyl-CoA dehydratase activity.

47. A method of producing a polypeptide, comprising culturing the cell of claim 45
5 under conditions that allow said cell to produce said polypeptide, wherein said polypeptide is produced.
48. A method for making 3-HP, said method comprising culturing at least one cell
10 comprising at least one exogenous nucleic acid molecule that encodes at least one polypeptide that is capable of producing said 3-HP from PEP under conditions such that said 3-HP is produced.
49. The method of claim 48, wherein said cell is selected from the group consisting of
15 yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
50. The method of claim 48, wherein 3-HP is made by a biosynthetic route that
utilizes a β -alanine intermediate.
51. The method of claim 48, wherein 3-HP is made by a biosynthetic route that
20 utilizes a malonyl-CoA intermediate.
52. The method of claim 48, wherein 3-HP is made by a biosynthetic route that
utilizes a lactate intermediate.
53. A method for making 3-HP, said method comprising culturing at least one cell
25 comprising at least one exogenous nucleic acid molecule that encodes at least one polypeptide that is capable of producing said 3-HP from lactate under conditions such that said 3-HP is produced.
54. The method of claim 53, wherein said cells are selected from the group consisting
30 of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

55. A method for making 3-HP, said method comprising culturing at least one cell under conditions wherein said cell produces said 3-HP, said cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.

5

56. The method of claim 55, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

57. The method of claim 55, wherein said cell comprises CoA transferase activity.

10

58. The method of claim 55, wherein said cell comprises 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.

59. A method for making 3-HP, said method comprising:

15

a) contacting lactate with a first polypeptide having CoA transferase activity to form lactyl-CoA,

b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA,

c) contacting said acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-HP-CoA, and

20

d) contacting said 3-HP-CoA with said first polypeptide to form said 3-HP or with a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form said 3-HP.

25 60. A method for making polymerized 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said polymerized 3-HP, said cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.

61. The method of claim 60, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

30

62. The method of claim 60, wherein said cell comprises CoA synthetase activity.
63. The method of claim 60, wherein said cell comprises poly hydroxyacid synthase activity.
- 5 64. A method for making polymerized 3-HP, said method comprising:
- a) contacting lactate with a first polypeptide having CoA synthetase activity to form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA
 - 10 dehydratase activity to form acrylyl-CoA,
 - c) contacting said acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA, and
 - d) contacting said 3-hydroxypropionic acid-CoA with a fourth polypeptide having poly hydroxyacid synthase activity to form said polymerized 3-HP.
- 15 65. A method for making an ester of 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said ester, said cell comprising lactyl-CoA dehydratase activity and 3-hydroxypropionyl-CoA dehydratase activity.
- 20 66. The method of claim 65, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
67. The method of claim 65, wherein said cell comprises CoA transferase activity.
- 25 68. The method of claim 65, wherein said cell comprises 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity.
69. A method for making an ester of 3-HP, said method comprising:
- a) contacting lactate with a first polypeptide having CoA transferase activity to
 - 30 form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA

dehydratase activity to form acrylyl-CoA,

c) contacting said acrylyl-CoA with a third polypeptide having 3-hydroxypropionyl-CoA dehydratase activity to form 3-hydroxypropionic acid-CoA,

d) contacting said 3-hydroxypropionic acid-CoA with said first polypeptide to
5 form 3-HP or a fourth polypeptide having 3-hydroxypropionyl-CoA hydrolase activity or 3-hydroxyisobutryl-CoA hydrolase activity to form 3-HP, and

e) contacting said 3-HP with a fifth polypeptide having lipase activity to form said ester.

10 70. A method for making polymerized acrylate, said method comprising culturing a cell under conditions wherein said cell produces said polymerized acrylate, said cell comprising CoA synthetase activity and lactyl-CoA dehydratase activity.

71. The method of claim 70, wherein said cell is selected from the group consisting of
15 yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

72. The method of claim 70, wherein said cell comprises poly hydroxyacid synthase activity.

20 73. A method for making polymerized acrylate, said method comprising:

a) contacting lactate with a first polypeptide having CoA synthetase activity to form lactyl-CoA,

b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA dehydratase activity to form acrylyl-CoA, and

25 c) contacting said acrylyl-CoA with a third polypeptide having poly hydroxyacid synthase activity to form said polymerized acrylate.

74. A method for making an ester of acrylate, said method comprising culturing a cell under conditions wherein said cell produces said ester, said cell comprising CoA
30 transferase activity and lactyl-CoA dehydratase activity.

75. The method of claim 74, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
76. The method of claim 74, wherein said cell comprises lipase activity.
- 5 77. A method for making an ester of acrylate, said method comprising:
- a) contacting lactate with a first polypeptide having CoA transferase activity to form lactyl-CoA,
 - b) contacting said lactyl-CoA with a second polypeptide having lactyl-CoA
 - 10 dehydratase activity to form acrylyl-CoA,
 - c) contacting said acrylyl-CoA with said first polypeptide to form acrylate, and
 - d) contacting said acrylate with a third polypeptide having lipase activity to form said ester.
- 15 78. A method for making 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said 3-HP, said cell comprising at least one exogenous nucleic acid that encodes at least one polypeptide such that said 3-HP is produced from acetyl-CoA and under conditions such that said 3-HP is produced.
- 20 79. The method of claim 78, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
80. A method for making 3-HP, said method comprising culturing a cell under conditions wherein said cell produces said 3-HP, said cell comprising at least one
- 25 exogenous nucleic acid that encodes at least one polypeptide such that said 3-HP is produced from malonyl-CoA and under conditions such that said 3-HP is produced.
81. The method of claim 80, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
- 30 82. A method for making 3-HP, said method comprising culturing a cell under

conditions wherein said cell produces said 3-HP, said cell comprising at least one exogenous nucleic acid that encodes at least one polypeptide such that said 3-HP is produced from β -alanine and under conditions such that said 3-HP is produced.

5 83. The method of claim 82, wherein said cell is selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

84. A method for making 3-HP, said method comprising culturing cells comprising an exogenous nucleic acid that encodes polypeptides that are capable of producing 3-HP
10 from acetyl-CoA under conditions such that said 3-HP is produced.

85. The method of claim 84, wherein said cells are selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.

15 86. A method for making 3-HP, said method comprising culturing cells comprising at least one exogenous nucleic acid that encodes polypeptides that are capable of producing said 3-HP from malonyl-CoA, and under conditions such that said 3-HP is produced.

87. The method of claim 86, wherein said cells are selected from the group consisting of yeast, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Escherichia* cells.
20

88. A method for making 3-HP, said method comprising:

a) contacting acetyl-CoA with a first polypeptide having acetyl-CoA carboxylase activity to form malonyl-CoA, and

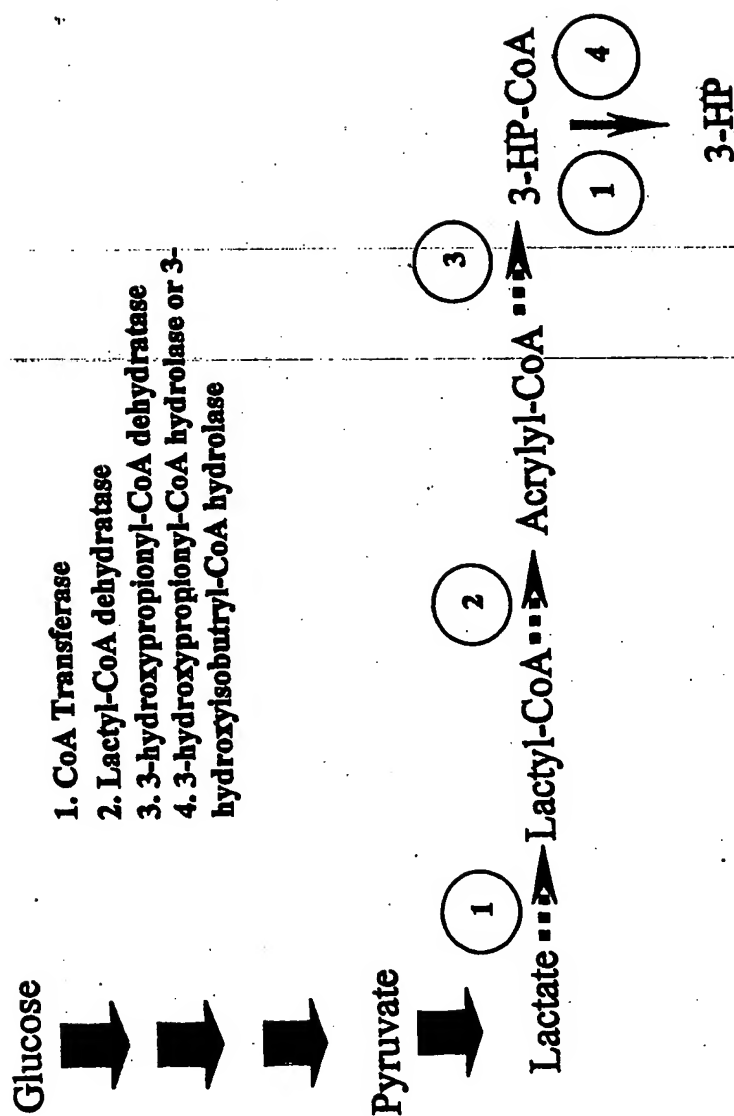
25 b) contacting said malonyl-CoA with a second polypeptide having malonyl-CoA reductase activity to form said 3-HP.

89. A method for making 3-HP, said method comprising contacting malonyl-CoA with a polypeptide having malonyl-CoA reductase activity to form said 3-HP.
30

90. A method for making 3-HP, said method comprising:

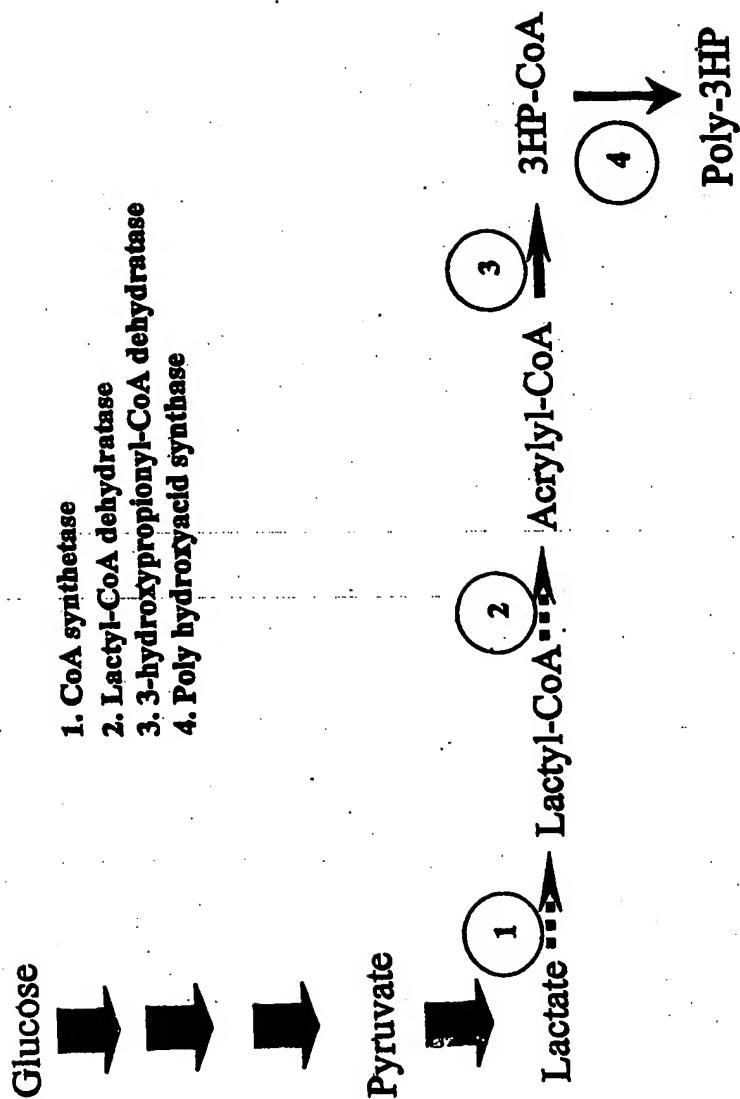
- a) contacting β -alanine CoA with a first polypeptide having β -alanyl-CoA ammonia lyase activity to form acrylyl-CoA;
- b) contacting said acrylyl-CoA with a second polypeptide having 3HP-CoA dehydratase activity to form said 3-HP-CoA; and
- 5 c) contacting 3-HP-CoA with a third polypeptide having glutamate dehydrogenase to make 3-HP.

1/105

Figure 1

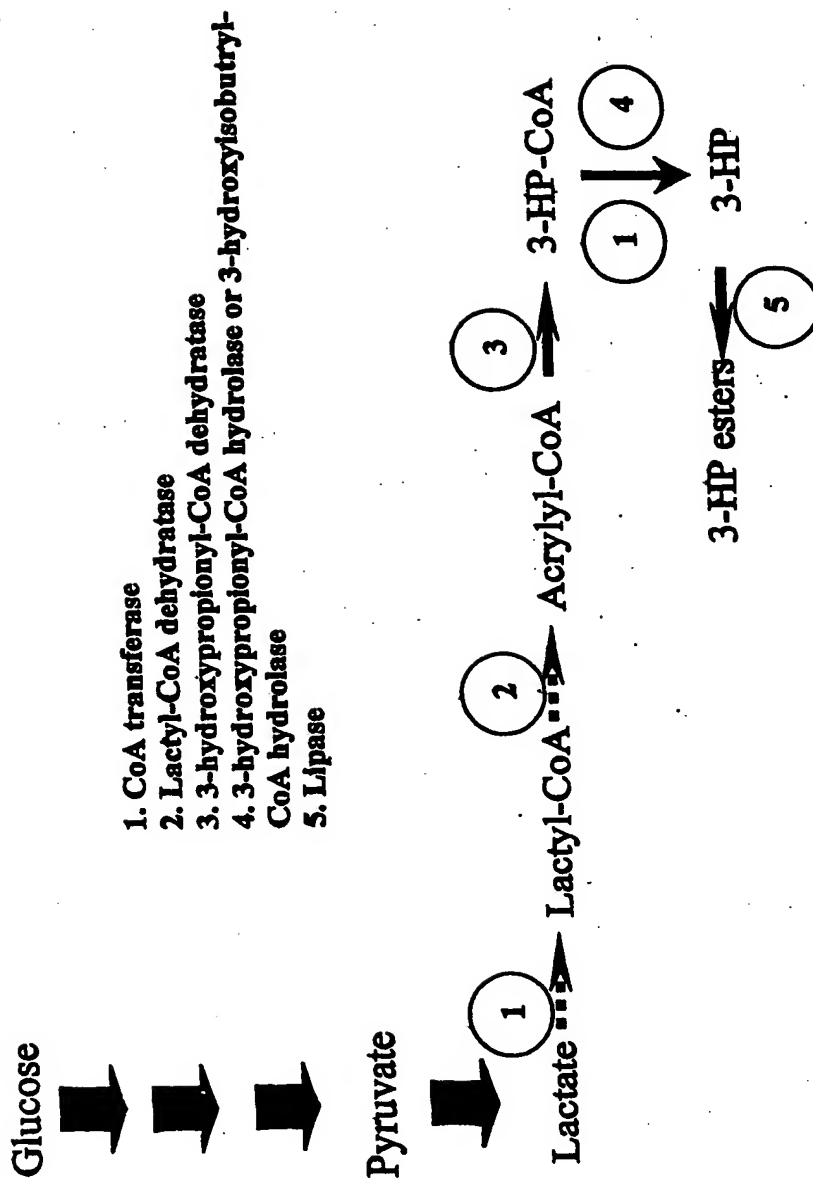
SUBSTITUTE SHEET (RULE 26)

2/105

Figure 2

3/105

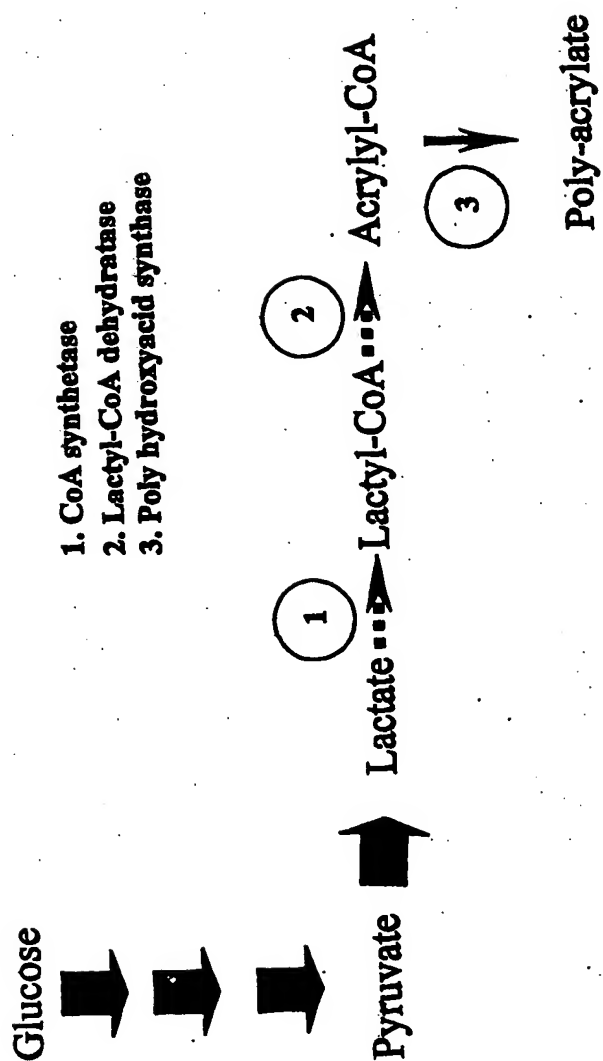
Figure 3



SUBSTITUTE SHEET (RULE 26)

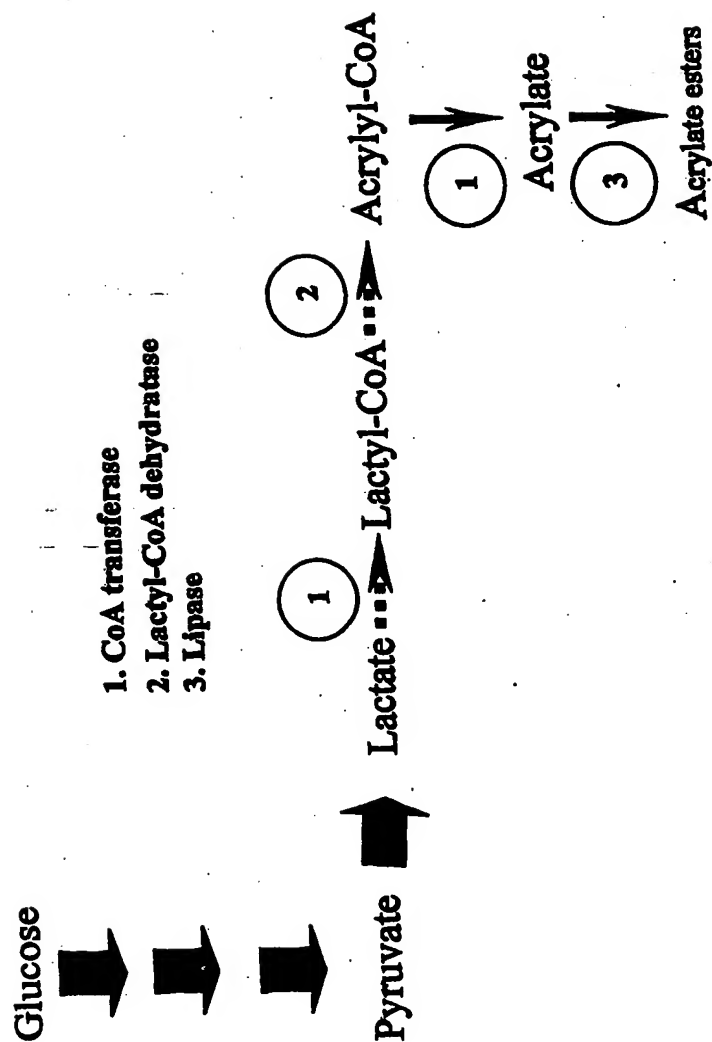
4/105

Figure 4



5/105

Figure 5



SUBSTITUTE SHEET (RULE 26)

6/105

Figure 6

ATGAGAAAAGTAGAAATCATTACAGCTGAACAAGCAGCTCAGCTCGTAAAAAGACAACGAC
ACGATTACGTCTATCGGCTTTGTGACGAGCGCCCATCCGGAAGCACTGACCAAAGCTTTG
GAAAAACGGTTCCTGGACACGAACACCCCGCAGAACTTGACCTACATCTATGCAGGCTCT
CAGGGCAAACGGGATGGCCGTGCCGTGAACATCTGGCACACACAGGCCTTTTGAAACGC
GCCATCATCGGTCACTGGCAGACTGTACCGGCTATCGGTAAACTGGCTGTCGAAAACAAG
ATTGAAGCTTACAACCTCTCGCAGGGCACGTTGGTCCACTGGTTCGCGCCTTGGCAGGT
CATAAGCTCGGCGTCTTACCGACATCGGTCTGGAACTTTCTCGATCCCCGTGACGCTC
GGCGGCAAGCTCAATGACGTAACCAAAGAAGACCTCGTCAAAGTATCGAAGTCGATGGT
CATGAACAGCTTTTCTACCGACCTTCCCGGTCAACGTAGCTTTCTCCGCGGTACGTAT
GCTGATGAATCCGGCAATATCACCATGGACGAAGAAATCGGGCCTTTGAAAGCACTTCC
GTAGCCAGGCGGTTCAAACTGTGGCGGTAAAGTCGTCTCCAGGTCAAAGACGTCTGTC
GCTCACGGCAGCCTCGACCCGCGCATGGTCAAGATCCCTGGCATCTATGTCGACTACGTC
GTGCTAGCAGCTCCGGAAGACCATCAGCAGACGTATGACTGCGAATACGATCCGTCCCTC
AGCGGTGAACATCGTGCTCCTGAAGGCGCTACCGATGCAGCTCTCCCATGAGCGCTAAG
AAAATCATCGGCCCGCGCGGCGCTTTGGAATTGACTGAAAACGCTGTCGTCAACCTCGGC
GTCGGTGCTCCGGAATACGTTGCTTCTGTTGCCGGTGAAGAAGGTATCGCCGATAACATT
ACCCTGACCGTTCGAAGGTGGCGCCATCGGTGGCGTACCGCAGGGCGGTGCCGCTTCGGT
TCGTCCCGCAATGCCGATGCCATCATCGACCACACCTATCAGTTCGACTTCTACGATGGC
GGCGGTCTGGACATCGCTTACCTCGGCCTGGCCAGTGCGATGGCTCGGGCAACATCAAC
GTCAGCAAGTTCGGTACTAACGTTGCCGGCTGCCGGCTTTCCCAACATTTCCCAGCAG
ACACCGAATGTTTACTTCTGCGGCACCTTCACGGCTGGCGGCTTGAAAATCGCTGTCGAA
GACGGCAAAGTCAAGATCCTCCAGGAAGGCAAAGCCAAGAAGTTTCATCAAAGCTGTCGAC
CAGATCACTTTCAACGGTTCCTATGCAGCCCGCAACGGCAAACACGTTCTCTACATCACA
GAACGCTGCGTATTTGAACTGACCAAAGAAGGCTTGAAACTCATCGAAGTCGCACCGGGC
ATCGATATTGAAAAAGATATCCTCGCTCACATGGACTTCAAGCCGATCATTGATAATCCG
AAACTCATGGATGCCCGCCTCTTCCAGGACGGTCCCATGGGACTGAAAAATAA (SEQ
ID NO:1)

7/105

Figure 7

MRKVEIITAEQAAQLVKDNDTITSIGFVSSAHPEALTKALEKRFLDTNTPQNLTYIYAGS
QGKRDGRAAEHLAHTGLLKRAIIGHWQTVPAIGKLAVENKIEAYNFSQGLVHWFRAIAG
HKLGVFTDIGLETFLDPRQLGGKLNVDTKEDLVKLI EVDGHEQLFYPTFPVNVAFIRGT
ADESGNITMDEEIGPFESTSVAQAVHNCGGKVVVQVKDVVAHGS LDP RMVKIPGIYVDYV
VVAAPEDHQQTYDCEYDPSLSGEHRAPEGATDAALPMSAKKIIGRRGALELTENAVVNLG
VGAPEYVASVAGEEGIADTITLTVEGGAIGGVPQGGARFGSSRNADAIIDHTYQDFDYDG
GGLDIAYLGLAQCDGSGNINVSKFGTNVAGCGGFPNISQOTPNVYFCGTFTAGGLKIAVE
DGKVKILQEGKAKKFIKAVDQITFNGSYAARNGKHVLYITERCVFELTKEGLKLI EVAPG
IDIEKDILAHMDFKPIIDNPKLMDARLFQDGPMGLKK (SEQ ID NO:2)

SUBSTITUTE SHEET (RULE 26)

8/105

Figure 8

SEQ ID NO:1	1 atgagaaaagtagaaatcattacagctgaacaagcagctc--agctcgta
SEQ ID NO:3	1 -----gtgccggtcctgtcggcacaggaagcggtga--attatatt
SEQ ID NO:4	1 atgccgattctctcaaaaatatggcggtccagcagctggaatcttgag
SEQ ID NO:5	1 -----atgaa-----tgca
SEQ ID NO:1	49 aaagacaacgacacgattacgtctatcggtttgtcagcagcgccatcc
SEQ ID NO:3	40 cccgacgaagcaacactttgtgtgttaggcgtg---gcggcggtattct
SEQ ID NO:4	51 aaaaactccgagaaatgctcatcaaatgaggtaaatctcaatga-catcc
SEQ ID NO:5	10 aaaga-----atta-----atcg-----
SEQ ID NO:1	99 ggaagcactgaccaagctttggaaaaacggttcctg-----
SEQ ID NO:3	87 ggaag-----ccaccacgtt--aattactgctcttgctgataaatataa
SEQ ID NO:4	100 tcgatgaaagcaaaagtcttt-----aactctgc-----
SEQ ID NO:5	23 -----
SEQ ID NO:1	136 ---gacacgaacaccccgagaaacttgacctacatctatgcag-gctctc
SEQ ID NO:3	129 acagactcaaacaccacgt--aattatcgattattagtccaa-cagggc
SEQ ID NO:4	129 -----cgaagaagccgtgaaggatattccagat-aatgcaaaagcttt
SEQ ID NO:5	23 -----ctcgccgaatt-----
SEQ ID NO:1	182 agggcaaacgcgatggcgtgcccgtgaacatctggcacacacaggcctt
SEQ ID NO:3	176 ttggcgatcgcccgaccgtgtattagctcttggcgcaagaaggtctg
SEQ ID NO:4	171 a-----gttggc--ggcttcggactatgcgg-aatccagaaaaat
SEQ ID NO:5	34 -----gcgatgg-----
SEQ ID NO:1	232 ttgaaacgcgccatcatcggtcactggcagactgtaccggc-tatcggt
SEQ ID NO:3	226 gtgaaatgggcattatgtggtcactgg-ggacaatcgccgctatttctg
SEQ ID NO:4	208 ctcatccaagctatca-caaaaactgggtcaa-----aaaggtc
SEQ ID NO:5	41 -----aattacatgatgga--ga-tattgtta
SEQ ID NO:1	281 aactggctgtcgaaaacaagattgaagcttacaacttctcgagggcacg
SEQ ID NO:3	275 aactcgagaaacaaaataaattattgcttataactaccacaagggtga
SEQ ID NO:4	245 ttacatgtgtatcaaacatgcgggagttgataatt-----ggggac-
SEQ ID NO:5	65 atctcgggt-----attg--gtttac-----caacacag
SEQ ID NO:1	331 ttggtccactggttcgcgccttgccaggtcataagctcgccgtcttcac
SEQ ID NO:3	325 cttacacaaaccttacgcgcgcgcagccaccagcctggtattattag
SEQ ID NO:4	287 ttggttgcctcttc--aaactcgacaaatc--aagaaaatgatctcatc
SEQ ID NO:5	92 ttgt-----taattattacctgataatgtcaata-----ttac
SEQ ID NO:1	381 cgacatcggtct----ggaaa----ctttcctcgatcccgctcagctcgcc
SEQ ID NO:3	375 tgatattggcat----cgga----catttgcgatccacgccagcaaggc
SEQ ID NO:4	333 gtacgtcggtgaaaacggaga--atttgcgcga--caatatcttagc
SEQ ID NO:5	126 --acttcaatca--gaaaatggctttcttggtttaactgca-----
SEQ ID NO:1	424 ggcaagctcaatgacgtaacca-----aagaagacctcgtaaaactgat
SEQ ID NO:3	418 ggcaaaactgaatgaagtacta-----aagaagacctgattaaactggt
SEQ ID NO:4	376 ggagagctcgagttggaattcacaccacaaggaaactcgccgaacgaat
SEQ ID NO:5	163 -----tttgac-----cca-----gaaaatgctaattcaaaact--
SEQ ID NO:1	468 cgaagtcgatgggtca---tgaacagcttttctaccggacc-----
SEQ ID NO:3	462 cgagtttgataacaa---agaatatctctattacaagcg-----
SEQ ID NO:4	426 tcgtgcagctggtgcccgtgttcccgcatctacac-accaacaggatac
SEQ ID NO:5	191 --tagtaaatgctgg---tggtcagcctt-----

9/105

SEQ ID NO:1	505	--ttcccg--tcaacgtagctttccctccggtacgtatgctga--tg
SEQ ID NO:3	499	--attgcgc--cagatattgccttcattcgcgctaccacctgcga--ca
SEQ ID NO:4	475	ggtaccagattcaagaaggaggtgctccga-ttaagtacagtaaaactg
SEQ ID NO:5	215	-----gtggaa-----ttaa--aa
SEQ ID NO:1	548	aatccggcaatatic-accatggacg-----aagaaatcgggcctttc
SEQ ID NO:3	542	gtgaaggctacgcc-acttttgaag-----atgaggtgatgtatctc
SEQ ID NO:4	524	aaaaaggaaagattgaagttgcaagtaaagcgaagaaacacgacaattc
SEQ ID NO:5	227	aaggcggctcta-----ctttt
SEQ ID NO:1	589	ga---aagcacttccgta---gccagggcgttcac--aactgtggcggt
SEQ ID NO:3	583	ga-----cgcatctggttattgccagggcggtgcac--aataacggcggt
SEQ ID NO:4	574	aatggaattaattatgtaatggaagaggtatttggggagatttgcatt
SEQ ID NO:5	244	ga---tagtgctt-----t--ttcttctcgcttc
SEQ ID NO:1	631	aaagtcgtcgtccaggtcaaagacgtcgtcgc-----tcacggcagocctc
SEQ ID NO:3	625	attgtgatgatgcaggtgcagaaaaagggttaa-----gaaagccacgctg
SEQ ID NO:4	624	gatcaaggcgtggagagcagatac-tcttggaatattcaattcagacat
SEQ ID NO:5	267	aa-----ttc
SEQ ID NO:1	676	gacccgcgcattgggtcaagatccctg-----gcatttatgtcgactac
SEQ ID NO:3	670	catcctaaatctgtccgtattccgg-----g--ttatctggtggat
SEQ ID NO:4	673	gctgctggaatttcaataatccaatgtgcaaaagcctctaaatgcac--c
SEQ ID NO:5	272	gtggcggtcatggt--gatgcctg-----tgtgctaggtggact--
SEQ ID NO:1	718	gtcgtcgtagcagctccggaagaccatcagcag--acgtatgactgcgaa
SEQ ID NO:3	709	attgtggtggtcgatccg--gatcaaacccaa--ctgtatggcggtgca
SEQ ID NO:4	721	atcgtcgaagtag--aggaaatcgtcgaaccgggagtaattgtctccaaa
SEQ ID NO:5	309	-----
SEQ ID NO:1	766	t-----acgatccgtccctcagcggtgaacatcgtgctcctg-aaggc
SEQ ID NO:3	754	c-----cggttaaccgctttatttctggtgacttcacccttg-atgac
SEQ ID NO:4	768	cgatgtgcacattccatcaatctattgtcatcgtctagtttgggaaaga
SEQ ID NO:5	309	-----tg-aagtt
SEQ ID NO:1	808	gctac-----cgatgcagc-----tctcccatgagcgctaaga
SEQ ID NO:3	796	agtac-----caaaacttag-----cctgccctaaac-caacgt
SEQ ID NO:4	818	actacaaaaaaccaatcgaacggccaatgttcgcacacgaaggaccaata
SEQ ID NO:5	316	gatca-----agaagcaaa-----tctcgc
SEQ ID NO:1	842	aaatcatcggc-cgcccggcgctttggaattgactgaaaacgctgtcgt
SEQ ID NO:3	829	aaattagttgcgcggcgcgctatttcgaaatgcgtaaaggcggtggg
SEQ ID NO:4	868	aaaccatctac-atcggc--tgctggaaaatcgagagaaatcattg-cag
SEQ ID NO:5	336	-----taactgga-----
SEQ ID NO:1	891	caacttcggcggtcggtgctcc-----ggaat--acgttgcttctgttgcc
SEQ ID NO:3	879	gaatgtcggcggtcggtattgc-----tgacg--gcattggcctggtcgcc
SEQ ID NO:4	914	cacgtgcagctttggagttcacagatggaatgtacgccaatttgggtatc
SEQ ID NO:5	344	-----tggtgac
SEQ ID NO:1	934	gg--tgaagaaggtatcgccga-----tacca-----ttaccctgac
SEQ ID NO:3	922	cg--agaagaaggttggtgctga-----tgact-----ttattctgac
SEQ ID NO:4	964	gggattccgactttggcgccaaattatataccaaatggatttactgttca
SEQ ID NO:5	351	tg--gcaaaatggta-----
SEQ ID NO:1	969	cgtcgaaggtg-----gcgccatcgttggtg--accgcagggcggtgcc
SEQ ID NO:3	957	ggtagaaacag-----gtccgattggcggaattacttcacaggggatcg
SEQ ID NO:4	1014	tttgcaagtgagaatgggtatttattggagtggtg--accata-----tcca
SEQ ID NO:5	364	-----

SUBSTITUTE SHEET (RULE 26)

10/105

SEQ ID NO:1	1012	cgcttcggttcgtcccgca-atgccgatgccatca----tcgaccacacc
SEQ ID NO:3	1001	c-ctttggcgcgaacgtga-ataccctgcccattc----tggatatgacg
SEQ ID NO:4	1057	agaaaag----gaacagaagacgccgatctcattaatgtcggaaaagagc
SEQ ID NO:5	364	-----ccagga-atg-----
SEQ ID NO:1	1057	tatcagttcgacttctacgatggcggc-----ggtctggacatcg
SEQ ID NO:3	1045	tcccagtttgatttttatcacggtggc-----ggtctggatggtt
SEQ ID NO:4	1103	---caattactcttct-caaaggagcttcaattgttggttctgatgaatc
SEQ ID NO:5	373	-----ggcgga-----gcaatggacttag
SEQ ID NO:1	1097	cttacctcggcctgg-----ccagtgctgatg-----gctcgggcaac
SEQ ID NO:3	1085	gttattttgagttttg-----ctgaagtcgacc-----agcacggtaac
SEQ ID NO:4	1149	attcgcaatgatctcgtggttctcatatggatattactgtgctcggtgcaac
SEQ ID NO:5	392	-----tg-----actggtgcaa-
SEQ ID NO:1	1135	atcaacgtcagca-agttcgggtactaacggttgcgggctcggcggtttcc
SEQ ID NO:3	1123	gtcggcgtgcata-aattcaatggtaaaatcatgggcacccggtggattta
SEQ ID NO:4	1199	ttca--gtgctcacagtttg--agatttagcgaattggatgattccg
SEQ ID NO:5	404	-----
SEQ ID NO:1	1184	ccaacatt--tcccagcagacaccgaatgtttacttctgcggcacct--tc
SEQ ID NO:3	1172	ttgatatacgtgccacttcgaagaaaatcatt--ttctgcggcacat--ta
SEQ ID NO:4	1243	ggaaaatt-----ggtga-aagggaatggcggtgcaatggatcttgtc
SEQ ID NO:5	404	-----aaaaagtgtattt-----ggca-----
SEQ ID NO:1	1231	acggctggcggttgaaaatcgctgtcgaagacggcgaagtcaagatcct
SEQ ID NO:3	1219	actgcgggcagtttataaacagaaattaccgacggcgaattaaatctcgt
SEQ ID NO:4	1285	tctgctcccg--agcccggtg-gatcgttgtaatggagcatgtat
SEQ ID NO:5	422	-----tggaacattg-----tgccaagtcaggttcct
SEQ ID NO:1	1281	ccaggaaggcaagccaagaagttcatcaaagctgtcgaccagatcactt
SEQ ID NO:3	1269	ccaggaaggacgggtgaagaaatttatccgggaactaccggaattactt
SEQ ID NO:4	1328	cgaagaacggagagccaaaaatt-----ctagagcactg
SEQ ID NO:5	449	caaaaattctaaag--aaatgtacattaccgct-----cacagcaagt
SEQ ID NO:1	1331	tcaacgg-----ttcctatgcagc---ccgcaacggcaaacacgttctct
SEQ ID NO:3	1319	tcagcggaaaaatcgctctcagc---gagggctgg-----atggtcgtt
SEQ ID NO:4	1362	cgaac-----ttcctctga--c---cggcaagg--agtaatttcccg
SEQ ID NO:5	490	aaaaaag-----ttgccatggtggttaccgaattggca-----gtattta
SEQ ID NO:1	1373	a--catcacagaacgctgcgtatttgaactgacca--aagaa-ggcttga
SEQ ID NO:3	1361	a--tatcactgagcgcgcagttatcacgctgaaag--aagac-ggcctgc
SEQ ID NO:4	1398	aatcattactgatatggcagttttcgacgtggacacaaagaacggattga
SEQ ID NO:5	530	a--cttcattgaaggcagattagtcta-----a--aagaa--catgc
SEQ ID NO:1	1418	aactcatcgaagtcgcaccgggcatcgatattgaaaaagatatcctcgt
SEQ ID NO:3	1406	atttaatcgaaatcgccctggcgtcgatttacaaaaagatatctcgac
SEQ ID NO:4	1448	cattgatcgaagt--caggaaggatc-ttactgtagatgatat-----
SEQ ID NO:5	567	tcctcat-----gtggatttagaaca--attaagoc
SEQ ID NO:1	1468	cacatggacttcaagccgat--cattgata--atccga--aactcatgg
SEQ ID NO:3	1456	aaaatggatttcacccaggt--gatttcgccagaactca--aactgatgg
SEQ ID NO:4	1488	--caagaaactca--ccg--cttgcaa--attcga--aatttccga
SEQ ID NO:5	598	aaaacag-----aagccgatttcattgtt-----gccgatgatttcaag
SEQ ID NO:1	1511	atgcccgctcttcaggacggtcccatggga-----ctgaaaaa--
SEQ ID NO:3	1502	acgaaagattatttatcgatgcggcgatgggtttgtcctgcctgaagcg
SEQ ID NO:4	1524	aaatctgaagccaatgggacagggtcctctta-----atcaaggataa-
SEQ ID NO:5	638	aaatgcaaatcagccag-----aaagga-----cttgaattatga

SUBSTITUTE SHEET (RULE 26)

11/105

SEQ ID NO:1	1552	-----taa
SEQ ID NO:3	1552	gctcattaa
SEQ ID NO:4	1567	-----
SEQ ID NO:5	673	-----

SUBSTITUTE SHEET (RULE 26)

12/105

Figure 9

SEQ ID NO:2	1	-----mrkveit-----aeqaaqlv
SEQ ID NO:6	1	-----mpvlis-----aqeavnyi
SEQ ID NO:7	1	mpilskiwaapaagilrktprnahqmrlistssmkakvfnsaeaeavkdi
SEQ ID NO:8	1	-----mnakeli-----arriamel
SEQ ID NO:2	17	knddtitsigfvssahpealt--kalekrfldtntpqnltyiyagsqgkr
SEQ ID NO:6	14	pdeaticvlg-agggileattlitaladkykqtqtpnlsiiisptglgdr
SEQ ID NO:7	51	pdnakllvggfglccgipenli--gai-----tktgqkgltcvsnnagv-
SEQ ID NO:8	16	hdgd-ivnlg-
SEQ ID NO:2	65	dgraaehlahtgllkraiighwqtvpaiqklavenkieaynfsqgtlvhw
SEQ ID NO:6	63	adrgisplaqeglvkwalcghwgqsprielaenkiiaynypqgvltqt
SEQ ID NO:7	92	dnwglglllqtrqikkmissyvgengefarqylsgeleleftpggtlaer
SEQ ID NO:8	25	-----
SEQ ID NO:2	115	fralaghklgvftdigletfldprqlggklnldvtkedlvkliev-----
SEQ ID NO:6	113	lraaaahqpgliisdigigtfdprqgggklnvtdkedliklvf-----
SEQ ID NO:7	142	iraagagvpafytpgygtqi---qeggapikysktekgk-ievaskake
SEQ ID NO:8	25	-----igl-----
SEQ ID NO:2	159	----dgheqlfyptfpvnvafirgtyadesgnitmdceigpfestsavaqa
SEQ ID NO:6	157	----dnkeylyykaiapdiafirattcdsegyatfedevmyldalviaqa
SEQ ID NO:7	188	trqfnginyvmeeaiwgdfalikawradtlgniqrhaagnfnnpmcakas
SEQ ID NO:8	28	-----ptqvvn-----yldpvnitlqsengflglta-----
SEQ ID NO:2	205	vhncggkvvvqvkdvhahgsldprmvkipgiyvdyvvaapedhqqtydc
SEQ ID NO:6	203	vhnnggiwmmqvqkmvkkatlhpksvripgyldv-ivvdpdqtqlygga
SEQ ID NO:7	238	---kc---tiveveiepgviapndvhipsiychrlvlg-----knykk
SEQ ID NO:8	55	-----
SEQ ID NO:2	255	eydpslsgehrapegatdaalpsakliigrrgaleltenavvnlgvg--
SEQ ID NO:6	252	pvnrfisgdfdl-ddstklslplnqrklvarrafemrkagvgnvgvg--
SEQ ID NO:7	277	pierpmfahegpikpstsaa--gksreilaaraaleftdgmnyanlgigip
SEQ ID NO:8	55	-fdp-----enansnl-vn--
SEQ ID NO:2	303	--apeyvasvageegiadtittlveggalg--gvpqgggarfgssrnad--
SEQ ID NO:6	299	--ladgiglvareregaddfilitvetgpig--gitsqgiafganvntr--
SEQ ID NO:7	325	tlapnyipn-----gftvhlqsengligvgpyprkgtedadlinagke
SEQ ID NO:8	67	--a-----ggqpc--gikkgstf-----
SEQ ID NO:2	347	-----aiddhtyqdfdydgggldiaylqlaqcdgsgni-nvskfgtn
SEQ ID NO:6	343	-----aiddmtsqdfdyhgglldvcylsfaevdqhgnv-gvhkfnkg
SEQ ID NO:7	368	pitllkgasivgsdesfamirgshmditvlgalcscqfgdlanwmipgkl
SEQ ID NO:8	82	-----dsafsfalirgghvdacvlgglevdqeanlanwmvpgkm
SEQ ID NO:2	388	vagcggfnpnisqqtpnvfyfcgtftagglkiav-----edgkvkilqegk
SEQ ID NO:6	384	imgtgffidisatskklifcgtltaglktei-----tdgklnivqegr
SEQ ID NO:7	418	vkmgggamdli-----vsapgarvivvmehvskngepkilehce
SEQ ID NO:8	121	vpmgggamdltvtgakkvii-----gmehca-----ksgsskilk--
SEQ ID NO:2	432	akkfikavdqitfngsyaarngkhvl--yitercvfel-tkegkklieva
SEQ ID NO:6	428	vkkfielpeitfsgkialergldvr--yiteravftl-kedgihlieia
SEQ ID NO:7	456	-----lpltgkgvisriitdmavfdvdtknigtlielivr
SEQ ID NO:8	155	-----kctlplt-----askkvam--vvtelavfnf-iegrlvleka

13/105

SEQ ID NO:2	479	pgidiekdi--lahmdfkpiidnp-klmdarlfqdgpmgkkl-----
SEQ ID NO:6	475	pgvdlqkdi--ldkmdftpvispelklmderlfidaamgfvlpeaah
SEQ ID NO:7	489	kdltvd-dikkltackfe-isenl-kpmgqaplnqg-----
SEQ ID NO:8	190	phvdle-ti--kakteadfvad-----dfkemqisqkglel-----

SUBSTITUTE SHEET (RULE 26)

14/105

Figure 10

GTGAAACTGTGTATACTCTCGGAATCGACGTTGGTTCTTCTTCTTCCAAGGCAGTCATC
CTGGAAGATGGCAAGAAGATCGTCGCCCATGCCGTGTTGAAATCGGCACCGGTTGACCC
GGTCCGGAACCGGTCTGGACGAAGTCTTCAAAGATACCAACTTAAAAATTGAAGACATG
GCGAACATCATCGCCACAGGCTATGGCCGTTTCAATGTCGACTGCGCCAAAGGCGAAGTC
AGCGAAATCACGTGCCATGCCAAAGGGGCCCTCTTTGAATGCCCCGGTACGACGACCATC
CTCGATATCGGCGGTGAGGACGTCAAGTCCATCAAATTGAATGGCCAGGGCCTGGTCATG
CAGTTTGCCATGAACGACAAATGCGCCGCTGGTACGGGCCGTTTCTCGACGTCATGTCG
AAGGTACTGGAAATCCCCATGTCTGAAATGGGGGACTGGTACTTCAAATCGAAGCATCCC
GCTGCCGTCAGCAGTACCTGCACGGTTTTTGCTGAATCGGAAGTCATTTCCCTTCTTTGC
AAGAATGTCCCGAAAGAAGATATCGTAGCCGGTGTCCATCAGTCCATCGCCGCCAAAGCC
TGCGCTCTCGTGCGCCGCGTCGGTGTGCGGTGAAGACCTGACCATGACCGGCGGTGGCTCC
CGCGATCCCGGCGTCGTGATGCCGTATCGAAAGAATTAGGTATTCTGTGAGAGTCGCT
CTGCATCCCCAAGCGGTGGTGCTCTCGGAGCTGCTTTGATTGCTTATGATAAAATCAAG
AAATAA (SEQ ID NO:9)

15/105

Figure 11

VKTVYTLGIDVGSSSSKAVILEDGKKIVAHAVVEIGTGSTGPVRLDEVFKDTNLKIEDM
ANIATGYGRFNVDCAKGEVSEITCHAKGALFECPGTTTTILDIGGQDVKSIKLNQGLVM
QFAMNDKCAAGTGRFLDVMSKVLEIPMSEMGDWYFKSKHPAAVSSTCTVFAESEVISLLS
KNVPKEDIVAGVHQSIKACALVRRVGVGEDLTMTGGGSRDPGVVDAVSKELGIPVRVA
LHPQAVGALGAALIAYDKIKK (SEQ ID NO:10)

SUBSTITUTE SHEET (RULE 26)

16/105

Figure 12

SEQ ID NO:9	1	gtgaaaactgtgtatactctcggaatcgacgttggttcttcttcttccaa
SEQ ID NO:11	1	---atgagtatctataccttgggaatcgatggttgatctactgcatbcaa
SEQ ID NO:12	1	gtggcagtgccatattcgattggcattgattccggctcaaccgccaccaa
SEQ ID NO:13	1	-----atgattttagggatagatggttgatctacaacacgcaa
SEQ ID NO:9	51	ggcagtcacacctggaagatggcaagaagatcgctgcg--ccatgccgctcggt
SEQ ID NO:11	48	gtgcattatcctgaaagatggaaaagaatcggtggc-gaaatccctggta
SEQ ID NO:12	51	agggatcttactggcagacggcggtgatta-----cgcgccgttctctcggt
SEQ ID NO:13	39	gatggttctaataggaagatagc---aagataatttg-gtataagatagag
SEQ ID NO:9	100	gaaatcggcaccggttcgaccggtccggaacgcgtcctggacgaagtctt
SEQ ID NO:11	97	gccgtggggaccggaacttccggtcccgacgggtctatttcggaagtcct
SEQ ID NO:12	97	ccaa-----ccccctttcgcccg--caacagcaattact---gaagcctg
SEQ ID NO:13	85	gatattgg-agttgtta-----ttgaggaagatattttatataaaatggtt
SEQ ID NO:9	150	caaagatacc-aacttaaaaattgaagacatggcgaaacatcatcgcg--cac
SEQ ID NO:11	147	ggaaaatgcc-cacatgaaaaagaagacatggcctttaccctggc-tac
SEQ ID NO:12	138	ggaa-actct-gcgcgaagggttagagacaacgcggtttctgacgctcac
SEQ ID NO:13	129	taaggagattgaacaaaaatatccaatagat----aaaatcgttgc-aac
SEQ ID NO:9	198	aggctatggccgtttcaatgtcg-----actgcgccaaggcggaag
SEQ ID NO:11	195	cggctacggacg---caat-tcgtggaaggcattggcgacaagcaga--
SEQ ID NO:12	186	cggctacggcgcgcaactggtg-----atgttgcgataaacagg
SEQ ID NO:13	174	tgatattggaaggcataaggtta-----gttttgagataagatag
SEQ ID NO:9	239	tcagcgaaatcacgtgccatgccaaaggggccc---ctctttgaatgcccc
SEQ ID NO:11	239	tgagcgaaactgagctgccatgccatggcgccc---agctttatctggccc
SEQ ID NO:12	227	taacggaaatctcctgtcacgggctggcgcca---cggtttcttgcgcca
SEQ ID NO:13	215	ttccagaagtta-ttgcattgggaaaaggagctaactatttctttaacga
SEQ ID NO:9	286	ggtacgacga--ccatcctcgatcggcggtcaggacgtcaa-gtccat
SEQ ID NO:11	286	--aacgtccataccgtcatcgatcggcggtcaggatgtgaa-ggtcat
SEQ ID NO:12	274	gcaacgcgcg--cggtaatcgacatcggtggtcaggacagcaaaagtgtt
SEQ ID NO:13	264	ggcagatgga----gttatagacattggagggcaagatacaaa-ggtctt
SEQ ID NO:9	333	caaattga--atggccagggcctggtcatgcagtttgcc-atgaacgaca
SEQ ID NO:11	333	ccatgttg--aaaacgggaccatgacca---atttccag-atgaatgata
SEQ ID NO:12	322	cagcttgatgatgacggttaacctg----tgcgatttcttgatgaatgaca
SEQ ID NO:13	309	aaagattg--ataaaaacggaaaagtgttgattttatc-ctatcagata.
SEQ ID NO:9	380	aatgcgccgctggtacgggcccgtttcctcgacgtcatgtcgaaggtactg
SEQ ID NO:11	377	aatgcgctgcgggactggccgtttcctggatgttatggccaatatcctg
SEQ ID NO:12	368	aatgcgcggcgccaccggcggtttcctggaggtgatctcgcgacgctt
SEQ ID NO:13	356	aatgtgccgctggaactggaaaattcttaga-----aaaggcatta
SEQ ID NO:9	430	gaaatccccatgtct-ga--aatgggggactggtactt-caaatcgaagc
SEQ ID NO:11	427	gaagtgaaggtttcc-ga--cctggctgagctgggagc-caaatccacca
SEQ ID NO:12	418	ggca--ccagcgtcgagc--aactcgacagcattaccg-aaaat---gtc
SEQ ID NO:13	397	gatattttaaaaatt-gataaaaatgagataaataaatacaaatcagata
SEQ ID NO:9	476	atccccgt-gccgtcagcagtagctgcacggtttttgctgaatcgggaag
SEQ ID NO:11	473	aacgggtg-gctatcagctccacctgtactgtgttgagaaaagtgaag
SEQ ID NO:12	460	acgcgcgacgcatcacaggtatgtgcacagtggttgctgaatcagaagc
SEQ ID NO:13	446	atatcgct-aaaatatcttcaatgtgtgtctcttctgctgaaagtgaag

17/105

SEQ ID NO:9	525 catttcccttctttccaagaatgtcccgaagaa--gatatcgtagccgg
SEQ ID NO:11	522 catcagccagctgtccaa--aggaaccgacaagatcgacatcattgccgg
SEQ ID NO:12	510 gatcagcctgcgctcagcggcgctcgccagaa--gcgattctcgagg
SEQ ID NO:13	495 aataagcttactatcaaaaaagttccaaaggaa--ggcattttaatggg
SEQ ID NO:9	573 tgtccatcagtcctcgcgcgcaagcctgcgctctcgtgc-gccgcgtc
SEQ ID NO:11	570 gatccatcgttctgtagccagcgggtcattggctcttgcca-atcgggtg
SEQ ID NO:12	558 agtgattaacgcgat-ggcgcggaggagtgc-caatttcat-tgctcgtc
SEQ ID NO:13	543 cgtctatgagagtat-----aataaatagggttatcccaatgaccaata
SEQ ID NO:9	622 ggtgtcgg--tgaagacctgaccatgaccggcggtggctcccgcgat--c
SEQ ID NO:11	619 gggattgt--gaaagacgtggtcatgaccggcggtgtagccagaac--t
SEQ ID NO:12	605 tctc-ctg--tgaagcgccgattctgtttactgggtggcgtagtcattgc
SEQ ID NO:13	587 ggcttaaaattcaaaacatagtgtttagtggaggagtgtctaaaaat--a
SEQ ID NO:9	668 ccggcgctcgtcgatgccgtatcgaaagaat-----taggtattcctgtc
SEQ ID NO:11	665 atggcgtgagaggagccct-----ggaag-----aaggccttggcggtg
SEQ ID NO:12	652 cagaagt-----ttgcccggtgctggaatctcacctgcgaatgccggtg
SEQ ID NO:13	635 aggttttggttgagatgtttgagaaaaaat-----tgaataaaaaacta
SEQ ID NO:9	712 agagtcgctctgcatccccaagcggtg-----ggtgctctcggagctgc
SEQ ID NO:11	703 gaaatcaagacgtctcccctggctcagtacaacgggtgccctgggtgccgc
SEQ ID NO:12	697 aatacccatcctgatgcgcaatttgct-----ggcgcaattggcgcggc
SEQ ID NO:13	679 ctaattccaaaagaaccacagattgtt-----tgctgtgtggagctat
SEQ ID NO:9	756 tttgattgctta-----tgataaatcaagaaa-taa
SEQ ID NO:11	753 tctgtatgcgta-----t-aaaaaagcagccaaataa
SEQ ID NO:12	741 ggtaattggtaacgagtgaggacacgccgatga---
SEQ ID NO:13	723 attggtt-----taa-----

SUBSTITUTE SHEET (RULE 26)

18/105

Figure 13

SEQ ID NO:10	1 vktvytlgidvgssskaviledgkkihavaveigtgstgpervldevf
SEQ ID NO:14	1 ms-iytlgidvgstaskciilkdgkeivakslvavgtgtsgparsisevl
SEQ ID NO:15	1 mavaysigidsgstatkgilladg-vitrflvpt---pfrpataiteaw
SEQ ID NO:16	1 ----milgidvgstttkmvmeds-kiiwykiedigv--viedillkmv
SEQ ID NO:10	51 kdtnlkiedmaniiatgygrfnvd-cakgevseitchakgalfecpgttt
SEQ ID NO:14	50 enahmkkedmafftlatgygrnslegiadqmselschamgasfiwvnt
SEQ ID NO:15	47 etlreglettpfltltygrqlvd-fadkqvteischglgarflapatra
SEQ ID NO:16	44 keieqkyp-idkivatgygrhkv-fadkivpeialgkanyffneadg
SEQ ID NO:10	100 ildiggqdvksiklnggglvmqfamndkcaagtgrfldvmskvlcpmse
SEQ ID NO:14	100 vidiggqdvkvihe-ngtmtntfqnmdkcaagtgrfldvmanilevkvd
SEQ ID NO:15	96 vidiggqdvskviqldddgnlcnldmndkcaagtgrflevisrtlgtveq
SEQ ID NO:16	92 vidiggqdvkvikidkngkvdfilsdkcaagtqgflekaldilkidkne
SEQ ID NO:10	150 mgdwyfkskhpaaavsstctvfaesevisllsknvpkedivagvhsiaak
SEQ ID NO:14	149 laelgakstkrvaisstctvfaesevisqlskgtdkidliagihrsvasr
SEQ ID NO:15	146 l-dsitenvtphaitsmctvfaeseaislragsvapeailagvinamarr
SEQ ID NO:16	142 ink--yksdniakissmcavfaeseisllskvpkegilmgvyesiinr
SEQ ID NO:10	200 acalvrrvgvgedltmtgggsrdpgvvdavskelgipvrvalhpqavgal
SEQ ID NO:14	199 viglanrvgivkdvmmtggvagnygvrgaleeglgveiktsplaqnygal
SEQ ID NO:15	195 sanfiarlsceapilftggvshcqqfarmleshlrmvpvntpdagfagai
SEQ ID NO:16	190 vipmtnrliki-qnivfsggvaknkvlvemfeklnkklilipkepqiavccv
SEQ ID NO:10	250 gaaliaydkikk--
SEQ ID NO:14	249 gaalyaykkaak--
SEQ ID NO:15	245 gaavig-qvrtrr
SEQ ID NO:16	239 gailv-----

19/105

Figure 14

ATGAGTGAAGAAAAACAGTAGATATTGAAGCATGAGCTCCAAGGAAGCCCTTGGTTAC
TTCTTGCCGAAAGTCGATGAAGACGCACGTAAAGCGAAAAAAGAAGGCCGCCTCGTTTGC
TGGTCCGCTTCTGTCGCTCCTCCGGAATTCTGCACGGCTATGGACATCGCCATCGTCTAT
CCGGAAACTCACGCAGCTGGTATCGGTGCCCCTCACGGTGCTCCGGCCATGCTCGAAGTT
GCTGAAAACAAAGGTTACAACCAGGACATCTGTTCTTACTGCCGCGTCAACATGGGCTAC
ATGGAACTCCTCAAACAGCAGGCTCTGACAGGCGAAACGCCGGAAGTCTCAAAAACCTCC
CCGGCTTCTCCGATTCCCTTCCGGATGTTGTCTCACTTGCAACAACATCTGCAATACC
TTGCTCAAATGGTATGAAAACCTTGGCTAAAGAATTGAACGTACCTCTCATCAACATCGAC
GTACCGTTCAACCATGAATCCCTGTTACGAAACACGCTAAACAGTACATCGTCGGCGAA
TTCAAACATGCTATCAAACAGCTCGAAGACCTTTGCGGCCGCTCCCTTCGACTATGACAAA
TTCTTCGAAGTACAGAAACAGACACAGCGCTCCATCGCTGCCTGGAACAAAATCGCTACG
TACTTCCAGTACAAACCGTCGCCGCTCAACGGCTTCGACCTCTTCAACTACATGGGCCTC
GCCGTTGCTGCCCGCTCCTTGAACACTCTCGGAAATCACGTTCAACAAATTCCTCAAAGAA
TTGGACGAAAAAGTAGCTAATAAGAAATGGGCTTTCCGGTGAACGAAAAATCCCGTGTT
ACTTGGGAAGGTATCGCTGTCTGGATCGCTCTCGGCCACACCTTCAAAGAACTCAAAGGT
CAGGGCGCTCTCATGACTGGTTCCGCTTATCCTGGCATGTGGGACGTTTCCTACGAACCG
GGCGACCTCGAATCCATGGCAGAAGCTTATTCCTGACATACATCAACTGCTGCCTCGAA
CAGCGCGGTGCTGTTCTTGAAAAAGTTGTCCGCGATGGCAAATGCGACGGCTTGATCATG
CACCAGAACCGTTCCTGCAAGAACATGAGCCTCCTCAACAACGAAGGCGGCCAGCGCATC
CAGAAGAACCTCGGCGTACCGTACGTCATCTTCGACGGCGACCAGACCGATGCTCGTAAC
TTCTCGGAAGCACAGTTCGATAACCGCGTAGAAGCTTTGGCAGAAATGATGGCAGACAAA
AAAGCCAATGAAGGAGGAAACCACTAA (SEQ ID NO:17)

SUBSTITUTE SHEET (RULE 26)

20/105

Figure 15

MSEKTVDIESMSSKEALGYFLPKVDEDARKAKKEGRLVCWSASVAPPEFCTAMDIAIVY
PETHAAGIGARHGAPAMLEVAENKGYNQDICSYCRVNMGYMELLKQOALTGETPEVLKNS
PASPIPLPDVVLTCNNICNTLLKWYENLAKELNVPLINIDVPENHEFPVTKHAKQYIVGE
FKHAIKQLEDLCGRPFDDYDKFFEVOQQTORSIAAWNKIATYFQYKPSPLNGFDLFNYMGL
AVAARSLNYSEITFNKFLKELDEKVANKKWAFFGENEKSRTWEGIAVWIALGHTFKELKG
QGALMTGSAYPGMWDVSYEPGDLESMAEAYSRTYINCCLEQRGAVLEKVVRDGGKCDGLIM
HONRSCKNMSLLNNEGGQRIQKNLGVPIYIFDGDQTDARNFSEAQFDTRVEALAEMMADK
KANEGGNH (SEQ ID NO:18)

SUBSTITUTE SHEET (RULE 26)

21/105

Figure 16

SEQ ID NO:17	1 atgagtgaagaaaaaacagtagatattgaaagcatgagctccaaggaagc
SEQ ID NO:19	1 atg-----ccaaagacagta-----agccctggcggttcagg-----
SEQ ID NO:20	1 ----atgatgaaattaaag--gcaattgaaaagttga--tgcaa-----
SEQ ID NO:21	1 -----atgtcacttgtcaccga-----tcta--cccgc
SEQ ID NO:17	51 cctt---ggttacttcttgccgaaa--gtcgatgaagacgca-----c
SEQ ID NO:19	32 -cat---tgagagatgtagtgtgaaaagggttacagagaactg-----c
SEQ ID NO:20	37 -----aaatt-----cgcca--gtagaaaagaacagc-----t
SEQ ID NO:21	27 cattttcgatcagttct--ctgaag--ctcgccagacaggctttctcacc
SEQ ID NO:17	89 gta-aagcgaaaa-aagaaggccgctcggtt-gctgggtccgcttctgtc
SEQ ID NO:19	71 ggg-aaccgaaag-aaagaggagaaaaagtag-gctgggtcctcttc--ca
SEQ ID NO:20	63 atataagcaaaaagaagaaggtagaaaagttt--ttggaatgttctgtg
SEQ ID NO:21	73 gtc-atggatctc-aaggag--cgcggcattccgctgggt-----tgge
SEQ ID NO:17	136 gctcctccggaattctgcacggctatggacatcgccatcgtc--tatccg
SEQ ID NO:19	116 agttccctcgcaactggctgaatcttttcggctgcatgttggtatccg
SEQ ID NO:20	110 cct-----atgttcca-----atagaaat--aat--tt--tagcag
SEQ ID NO:21	112 act-----tactgcacctttatg----ccgcaagag-----atccc
SEQ ID NO:17	184 gaaactca--cgagctggtatcggtgcc--cgtcacggtg-----
SEQ ID NO:19	166 gaaaacca--ggctgctggtatcgctgccaaaccgtgacggcggaagtgatg
SEQ ID NO:20	140 caaatgcaatcccagttggtttgtgtgga--ggtaaaaaat-----
SEQ ID NO:21	144 ga----t--ggcagc-----cggtgcg--gtt--gtg-----
SEQ ID NO:17	221 -----ctccggccatgc
SEQ ID NO:19	214 tgccaggctgcagaagatatcggttatgacaacgatatctgcggctatgc
SEQ ID NO:20	178 -----gacacaa
SEQ ID NO:21	166 -----gtttcgctctgt
SEQ ID NO:17	233 tcgaagt-t-----gctg-----aaaa--
SEQ ID NO:19	264 ccgtatt-tccctggcttatgctgccgggttccggggtgccaaacaaatg
SEQ ID NO:20	185 tcccaat-a-----gcag-----a-----
SEQ ID NO:21	178 tccacctct-----gatg-----aaac--
SEQ ID NO:17	249 --caaaggttacaccaggacatctgttctactgccgcgtcaacatg--
SEQ ID NO:19	313 gacaaagatggcaactatgtcatcaacccccacagcggaacacagatgaa
SEQ ID NO:20	198 ---ggaggat-ttgcaagaacacatgccc-----cattaata----
SEQ ID NO:21	195 --ca--ttgaagaagcggagaaagat-----ctgccgcg-caacct--
SEQ ID NO:17	295 -----ggctacatggaactc--ctcaaacagcag-----
SEQ ID NO:19	363 agatgccaatggcaaaaaggtattcgacgcagatggcaaacccgtaatcg
SEQ ID NO:20	232 -----aaatc--atccta--tg-----
SEQ ID NO:21	231 -----ctgcccg--ctg--attaata-agca-----
SEQ ID NO:17	322 -----
SEQ ID NO:19	413 atcccaagaccctgaaaccctttgccaccaccgacaacatctatgaaatc
SEQ ID NO:20	245 -----
SEQ ID NO:21	251 -----
SEQ ID NO:17	322 ---gctctgac--aggcgaaa-----cgccggaa-gtccctcaa
SEQ ID NO:19	463 gctgctctgccggaagggaagaaaagaccgcgccgagaaatgccctgca
SEQ ID NO:20	245 ---gttttaa-----gaá-ggca--aa
SEQ ID NO:21	251 ---gctacggc--ttcggcaa-----aaccg-----at

SUBSTITUTE SHEET (RULE 26)

22/105

SEQ ID NO:17	354 aaactccccggcttctccgattccccctccggatgtgtgcctcacttgca
SEQ ID NO:19	513 caaatatcgatgacatgacatgcccagcttcgtgctgtgctgca
SEQ ID NO:20	261 aacctgccc--ttactttgaagcatct----gatatagttat-tggagaa
SEQ ID NO:21	274 aaatgcccttacttct-----acttttcggatctggtggtc--ggtg
SEQ ID NO:17	404 acaacatctgca-----ataccttgctcaaatggtatgaaaacttg-
SEQ ID NO:19	563 acaacatctgca-----actgcatgaccaaaggtatgaagacattg-
SEQ ID NO:20	304 actacctgtgaaggaaagaagatgtttgagttgatggagagattggt
SEQ ID NO:21	314 aaaccacctgcg-----acggcaaaaagaaaatgtatgaatacatgg-
SEQ ID NO:17	446 -ctaaagaattgaac---gtacctctca----tcaacatcgacgtac--c
SEQ ID NO:19	605 -cccgtcgccacaac---attcctttga----tcatgatcgacgttc--c
SEQ ID NO:20	354 gccaatgcatataat---gcacctccacacatgaagatgaagatt--c
SEQ ID NO:21	356 -c---ggagtttaagcctgttcattgtga----tgca-attgcccaacagc
SEQ ID NO:17	486 gttca--acatgaattc---cctg--tta-cgaa----ac--acgctaa
SEQ ID NO:19	645 ttaca--ac---gaattcgaccatg--tcaacgaa----gccaacgtgaa
SEQ ID NO:20	399 tttga--a-----aatct---ggat--taa-agaagttgaa--aagctaa
SEQ ID NO:21	397 gttaaggacgatgcctcg---cgtgcgtta-tgga-----a-----
SEQ ID NO:17	522 acagtacatcgctg-----gcgaattcaaacatgctatca----aacagc
SEQ ID NO:19	684 a---tacatccggt-----cccagctggatagcgccatcc----gtcaaa
SEQ ID NO:20	434 --aagaattggttgagaaagagactggaataaaaaaacagaggaaggt
SEQ ID NO:21	429 --agccgagatgct-----gcgcttgcaa-----a-----aacagg
SEQ ID NO:17	563 tcgaagacaccttgcggccgtcccttcgactatgacaaattcttcgaagta
SEQ ID NO:19	722 tggaagaaatcacccggcaagaagttcgatgaagacaaattc-----gaa
SEQ ID NO:20	482 taaaaga-----gacagttgat--aaagta
SEQ ID NO:21	458 tagaagaacggttttgggacagagattagcgaagatgctctgcgcgatgcc
SEQ ID NO:17	613 cagaacacagacacagcgctc-catcg--ctgcc-----tggaacaaaat
SEQ ID NO:19	766 cag-tgctgccagaacgc-c-aaccgtactgccaaagcatggctgaaggt
SEQ ID NO:20	505 aataaagttaggag-----t-----tgttttataaa
SEQ ID NO:21	508 attgcgctgaaaaaccgcgaacgtcg--cgcac-----tgg--ctaata
SEQ ID NO:17	654 cgctacgtacttc--c--agtacaaaccgtcgccgctcaacggcttcgac
SEQ ID NO:19	813 ttgcgactacctg--c--agtacaaaccggctccgttcaacgggttcgac
SEQ ID NO:20	532 ctctatgaattga--ggaagaataaaccaagctccaattaagggtttagat
SEQ ID NO:21	547 ttttatcatcttgggc--agttaaactcctccggcgcttagcgcagcgac
SEQ ID NO:17	700 ctcttcaactacatgggcctcgccg--ttgctgcocgctccttgaactact
SEQ ID NO:19	859 ctgttcaaccataggtgacgtgg--ttaccgcccgtggccgtgtggaag
SEQ ID NO:20	580 gttttaaattattccagtttgctatttattggatattgatgacacaat
SEQ ID NO:21	595 attctga---aagtgggttacggcg--caaccttccggttcgataaagagg
SEQ ID NO:17	749 cggaatcacgttcaacaaattcctcaaagaattggacgaaaaagtagc-
SEQ ID NO:19	908 ctgctgaagctttcgaactgctggccaagggaactggaacagcatgt---
SEQ ID NO:20	630 agggatt---ttagaggatttaattgaggagttagaggagagaggt---
SEQ ID NO:21	641 cg-----ttgatcaatgaactggatgcaatgaccgcc
SEQ ID NO:17	798 -----taataagaaatgggctttcggtgaa-----aacgaaaaatccccg
SEQ ID NO:19	954 -----gaagggaaggcaccaccacgctcccttcaaagaacagcatcg
SEQ ID NO:20	673 -----aaaaaaggagaagggttatgaaggaa-----agagaa-----
SEQ ID NO:21	673 cgcgttcgtcagcagtggaagaag--gcc-----agcgactggacccc
SEQ ID NO:17	837 tgttacttgggaaggtat--tcgctgtctggatcgctctcgccacacc---
SEQ ID NO:19	996 tatcatgtttcgaaggga--tcccctgctgg--ccgaaactgcccgaacc---
SEQ ID NO:20	704 -----ttttaataac-tggctgtc--caatgggttgcgtggaacaataag
SEQ ID NO:21	715 cgt--ccgcgcatttttaatacaccggctg---cccgattggcgccgc---

SUBSTITUTE SHEET (RULE 26)

23/105

SEQ ID NO:17 883 ----t--tcaaagaactca--aaggtcagggcgctctcatgactgggtcc
SEQ ID NO:19 1040 ----tggtcaaaccgctga--aagccaacggcctgaacatcacccggcgtt
SEQ ID NO:20 745 attgt--tgaaattattgaggaagtt---ggaggagtagttgttggtgaa
SEQ ID NO:21 756 -----agcaga--aaaagtgggtgcgcgcgattgaagagaatg

SEQ ID NO:17 925 gcttat---cctggcatgtgggacgtttcctacgaacc-----ggg-
SEQ ID NO:19 1084 gtatatgctcctgctttcgggttcgtgtacaacaacct-----gga-
SEQ ID NO:20 790 g---aaa---gctgcactggaacaagattctttgaaaactttgttgaggg-
SEQ ID NO:21 791 gc---g---gctgggttgctcggttatgaaaactgcacc-----gggg

SEQ ID NO:17 963 -----cga---cctcg-aatccatggcagaa----gcttattcccgtae
SEQ ID NO:19 1125 -----cga---attgg---tcaaagcctact----gcaaagcccgaac
SEQ ID NO:20 834 -----ctatagcgtag-aggacattgcaaaa--agata--cttta
SEQ ID NO:21 827 cgaaagcga---ccgagcaatgcgtggcagaaacgggcgatgtctacgac

SEQ ID NO:17 999 atac-----atcaactgctgcct-----cgaacagcgcggtgct
SEQ ID NO:19 1159 -tcc-----gtca-----gcat-----cgaacaggggtgttgcc
SEQ ID NO:20 869 aaat-----cccatgtgctttagatttaaaaacgatgagagagttgaa
SEQ ID NO:21 874 gcgctggcggataaataatctggc-----gattggctgctcct

SEQ ID NO:17 1033 gttcttgaaaaagttgtccgcgatggcgaatgcgacggc--ttgatcatgc
SEQ ID NO:19 1186 tggcgtgaaggcctgatccgcgacaacaaggttgacggc--gtactggttc
SEQ ID NO:20 913 aatataaagagattggttaaagagttggacgtcgatggagttgtttat--
SEQ ID NO:21 911 gtgttttgcgaacgatcagcgcctgaaaatgc--tcagc--cagatggttg

SEQ ID NO:17 1082 accagaacc-gttcctgcaagaacatgagcctcctcaacaacgaaggcg-
SEQ ID NO:19 1235 actacaacc-ggtcctgcaaaccttgagcggctacatgcctgaaatgc-
SEQ ID NO:20 961 ----tacac-tttgcagtattgccat----acatttaacatagagggagc
SEQ ID NO:21 959 aggaatatcaggtcgtatggcgtagtga----tgtgattttgcaggcgt

SEQ ID NO:17 1130 ---gocagcgcac--cagaagaacctc---ggcgtaccgtacgtcatcttc
SEQ ID NO:19 1283 ---agcgtcgtttc-accaaagacatg---ggtatccccactgctggattc
SEQ ID NO:20 1002 taaggtagaggagg-cattaaaagaggagggcattccaattataagaatt
SEQ ID NO:21 1004 ---gccatacctacgcggtggaatcgc--tggcgattaaacgtcatgtgc

SEQ ID NO:17 1174 gacggcgaccagaccgatgctcgtaacttctcggaagca-----
SEQ ID NO:19 1327 gacggtgaccaggctgacccgagaaacttcaacgcggct-----
SEQ ID NO:20 1051 gaaactgactattctga-----aagtgatag--agag-----
SEQ ID NO:21 1049 gccagc-agcacaacattccttatatcgctattgaaacagactactccac

SEQ ID NO:17 1213 -----cagttcgatacccgcgtagaagctttggcagaaatga
SEQ ID NO:19 1366 -----cagtatgagaccggtgttcagggttggtcgaagcca
SEQ ID NO:20 1081 -----cagttaaaaacaaggttgaggcattttattgagatga
SEQ ID NO:21 1098 ctcggatgtcgggcagctcagtaaccggtgcgcggcctttattgagatgc

SEQ ID NO:17 1250 tggcagacaaaaaagccaatgaaggaggaaaccactaa
SEQ ID NO:19 1403 tggag--caaagatgaaaagaagg--ggaataa-----
SEQ ID NO:20 1118 t-----ttaa-----
SEQ ID NO:21 1148 tgtaa-----

SUBSTITUTE SHEET (RULE 26)

24/105

Figure 17

SEQ ID NO:18	1 --mseektvdiessskeletalgyflpkvdedarkakkegrlvcwsasvapp
SEQ ID NO:22	1 ----mpktvs----pgvqalrdvvekvyrelrepkergekvwssskfpc
SEQ ID NO:23	1 --nmklka--ieklmqkfa-----srkeglykqkeegrkvfgm-----
SEQ ID NO:24	1 mslvtdlpaifdqfsearqtg-fltvmldkergiplvg-----
SEQ ID NO:18	49 efctamdlaivypethaag---igarhgapamlevaenkgynqdicyscr
SEQ ID NO:22	43 elaesfrlhvgypenqaag---iaanrdgevmcqaedigyndicgyar
SEQ ID NO:23	35 -fcayvpieilla-anaip---vglcggkndtipiae-edlprnlcpplik
SEQ ID NO:24	38 tyctfmpqei----pmaagavvvslcstsdetieeae-kdlprnlcpplik
SEQ ID NO:18	96 vnmgyg-----
SEQ ID NO:22	90 islayaagfrgankmdkdgnvynphsgkqmkdangkkvfdadgkpvidp
SEQ ID NO:23	79 ssygf-----
SEQ ID NO:24	83 ssygf-----
SEQ ID NO:18	102 ellkqaltgetpev-----lknspaspiplpdvvltcnn
SEQ ID NO:22	140 ktlkpfattdniyeialaalpegeektrrqnalhkylrqmtmpmpdfvlccnn
SEQ ID NO:23	84 -----kkaktcpyfeasdiviget
SEQ ID NO:24	88 -----gktdkcpyf-----y-----fsdlvvg-et
SEQ ID NO:18	137 icntlikwienlakelnvplnidvpfnhefpvtkhakqyivgefkhak
SEQ ID NO:22	190 icncmtkwyediarrhniplimidvpynfedhvnneanvkylrsqldtair
SEQ ID NO:23	103 tceggkkmfelm--erlvpmhlmhplhmkd----edsikiwikeveklike
SEQ ID NO:24	107 tcdgkkkmyeymaefkpvhvmqlpnsvkdd-----asralwkaemlrkq
SEQ ID NO:18	187 qledlcgrpfdydkffe---vqkqtqrsiaawnkiatyfyqkpsplngfd
SEQ ID NO:22	240 qmeeitgkkfdedkfeq---ccqnanrtakawlkvcldylqykpapfngfd
SEQ ID NO:23	147 lveketgnkiteeklike---tvdkvnkvrelfyklyelrknkpapikgld
SEQ ID NO:24	152 tveerfgheisedalrdaialknrrerlanfyhlq---qlnppalsgsd
SEQ ID NO:18	234 ---lfnymglavaarslnyseitfnkflkeldekvan--kkwafge--n-
SEQ ID NO:22	287 ---lfnhmadvvtargrveaaefellakeqhvke--gtttapf--k-
SEQ ID NO:23	194 vlklfqfaylldiddtigile----dlieeleerv---kk--ge--gy
SEQ ID NO:24	199 ---ilk---vvygatfrfdk---ealineldamtarvrqqweegqrld-
SEQ ID NO:18	276 eksrvtwegiavwialghtfkelkgqgalmtg----say---pgmwdvsv
SEQ ID NO:22	329 eqhrimfegipcwplpnlfkplkanglnitg----vvy---apafgfvv
SEQ ID NO:23	231 egkrilitgcpmvagnnkiveeieevggvvvg----eesctgtrffenf
SEQ ID NO:24	238 prprilitgcpiggaaekvvraieenggwvvgyenctga---kateqcva
SEQ ID NO:18	319 epgdil-esmaeayertyinccl--eqrgavlekvvrdgkcdglimhqns
SEQ ID NO:22	372 --nnl-delvkayckapnsvisi--eggvawreglirdnkvdgvlvhnrs
SEQ ID NO:23	277 egysv-ediakryfkipcacrfkndervenikrlvkeldvdgvytytlqy
SEQ ID NO:24	285 etgdvydaladkylaigscscvspndgrikmlsqmveeyqvdgvydvilqa
SEQ ID NO:18	366 cknmsallnnegg--griqknlgvpyvifdgdqtdarnfseaqfdtrveal
SEQ ID NO:22	417 ckpwsgympemq--rrftkdmgiptagfdgdqadprnfnaaqyetrvggl
SEQ ID NO:23	326 cht--fniegakveealkeegipiirietdyses---dreqlktrleaf
SEQ ID NO:24	335 chtyaveslaik--rhvrqqhnpiaiai---etdystsdvgqlstrvaaf
SEQ ID NO:18	414 aemmadkkaneggnh
SEQ ID NO:22	465 veameandekkgk--
SEQ ID NO:23	370 iemi-----
SEQ ID NO:24	380 ieml-----

25/105

Figure 18

ATGAGTCAGATCGACGAACCTTATCAGCAAATTACAGGAAGTATCCAACCATCCCCAGAAG
ACGGTTTTGAATTATAAAAAACAGGGTAAAGGCCTCGTAGGCATGATGCCCTACTACGCT
CCGGAAGAAATCGTATATGCTGCAGGCTACCTCCCGGTAGGCATGTTGGTTCCCAGAAC
CCGCAGATCTCCGCAGCTCGTACGTACCTTCTCCGTTGCTCCTTGATGCAGGCT
GACATGGAACCTCAGCTCAACGGCACCTATGACTGCCTCGAGGCTGTTATCTTCTCCGTT
CCTTGCGACACTCTCCGCTGCATGAGCCAGAAATGGCACGGCAAAGCTCCGGTCATGGTC
TTCACACAGCCGCAGAACCGTAAGATCCGCCCGGCTGTCGATTTCTCAAAGCTGAATAC
GAACATGTCCGTACGGAATTGGGACGTATCCTCAACGTA AAAATCTCCGACCTGGCTATC
CAGGAAGCTATCAAAGTATATAACGAAAACCGTCAGGTTATGCGTGAATTCTGCGACGTA
GCTGCTCAGTACCCGCAGATCTTCACTCCGATAAAACGTCATGACGTCATCAAAGCCCGC
TGGTTCATGGACAAAGCTGAACACACCGCTTTGGTCCGCGAACTCATCGACGCTGTCAAG
AAAGAACCGGTACAGCCGTGGAAATGGCAAAAAGTCATCTCTCCGGTATCATGGCAGAA
CCGGATGAATTCTCGATATCTTCAGCGAATTCACATCGCTGTGTCGCTGACGACCTC
GCTCAGGAATCCCGCCAGTTCGTACAGACGTACCGTCCGGCATCGATCCCCTCGAACAG
CTCGCTCAGCAGTGGCAGGACTTCGATGGCTGCCCGCTCGCTTTGAACGAAGACAAACCG
CGTGGCCAGATGCTCATCGACATGACTAAGAAATACATGCTGACGCCGTCGTCTATCTGC
ATGATGCGTTTCTGCGATCCTGAAGAATTCGACTATCCGATTTACAAACCGGAATTTGAA
GCTGCTGGCGTTCGTTACACGGTCTCGACCTCGACATCGAATCTCCGTCCCTCGAACAG
CTCCGCACCCGTATCCAGGCTTTCTCGGAAATCCTCTAA (SEQ ID NO:25)

SUBSTITUTE SHEET (RULE 26)

26/105

Figure 19

MSQIDELISKLQEVSNHPQKTVLNYKKQKGLVGMMPYYAPEEIVYAAGYLPVGMFGSQN
PQISAARTYLPPFACSLMQADMELQLNGTYDCLDAVIFSVPCDTLRCMSQKWHGKAPVIV
FTQPQNRKIRPAVDFLKAEYEHVRTELGRILNVKISDLAIQEAIKVYNENRQVMREFCDV
AAQYPQIFTPIKRHDVIKARWFMDKAEHTALVRELIDAVKKEPVQPNWGKKVILSGIMAE
PDEFLDIFSEFNIAVVADDLAQESRQFRTDVPSGIDPLEQLAQWQDFDGCPLALNEDKE
RGQMLIDMTKKYNADAVVICMMRFCDPEEFDYPIYKPEFEAAGVRYTVLDDLIESPSLEQ
LRTRIQAFSEIL (SEQ ID NO:26)

27/105

Figure 20

SEQ ID NO:25	1 atgagtcagatcgacgaacttatcagcaaattacaggaagtatccaacca
SEQ ID NO:27	1 atggct---atcagtgcaacttattgaagagttccaaaaagtat-ctgcca
SEQ ID NO:28	1 -----atgatgaaattaaaggcaattgaaaagtgtgatgcaaaaaat
SEQ ID NO:29	1 atgtcacttgtcaccgactctaccgccattttcgatcagttctctgaagc
SEQ ID NO:25	51 tccccagaag-----ac-----ggttttg---aattataaaaaa
SEQ ID NO:27	47 gcc---gaag-----ac-----catgctggccaaatataaaagcc
SEQ ID NO:28	41 tcgccagtag-----aaaagaacagctatat---aagcaaaaaagaa
SEQ ID NO:29	51 tcgccagacaggctttctcac-----cgctcatg---gatctcaaggag
SEQ ID NO:25	82 cagggtaaaggcctcgtaggca--tgatgccctactacgctccggaagaa
SEQ ID NO:27	79 cagggtcaaaaaagccatcggt--gcctgccgtactatgttccggaagaa
SEQ ID NO:28	79 gaaggtagaaaaagtttttgaa--tggtctgtgcctatgttccaatagaa
SEQ ID NO:29	91 cgcggcattccgctggttggaacttactgcacctttatgc--cgcaagag
SEQ ID NO:25	130 atcgtatatgctgcaggctacctcccggtaggcatgt---tcggttccca
SEQ ID NO:27	127 ctggtctatgctgcaggcatggttcccatgggtgtat---ggggtgcga
SEQ ID NO:28	127 ataattttagcagcaaatgcaatcccaagtgtggtttgt---gtggaggtaa
SEQ ID NO:29	139 atcccgatggcagccgg-----tgcggttgtggtttcgctctgttccac
SEQ ID NO:25	177 -----gaaccgcag-atctccgcagctcgtaaccttctcccggtt
SEQ ID NO:27	174 -----tggcaaacagggaagtccgttccaaggaa-tactgtgcttcctt
SEQ ID NO:28	174 -----aaatgacaca-atcccaatagcagaggaggatttgccaagaaa
SEQ ID NO:29	183 ctctgatgaaacc-----attgaagaagcggagaagaatctgcgcgcgcaa
SEQ ID NO:25	219 cgcttgcctccttgatgcaggctgacatggaactccagctcaacggca---
SEQ ID NO:27	216 ctactgcaccattgcccagcagctctctggaatgctgctggacggga---
SEQ ID NO:28	216 cctatgcccatataataaaatcatcctatggttttaag---aaggca---
SEQ ID NO:29	228 cctctgcccgctga-----ttaaagcagctacggct---tcggcaaaa
SEQ ID NO:25	266 cctatgactgcctcgacgctgttatcttctcc-----gttctt-tgcg---
SEQ ID NO:27	263 ccctggatgggttggaaggatcatca-ctcc-----ggtactgtgtg---
SEQ ID NO:28	259 ---aaaacctgcccttactttg-aagcatctgatatagttatt-ggaag---
SEQ ID NO:29	269 ccgataaatgccctac-----ttctacttttcc-----ggatct-ggtggtc
SEQ ID NO:25	308 ----acactctccgctgcatgagccagaaat-----gg-----c-
SEQ ID NO:27	305 ----ataccctgcgtcccatgagccagaaacttcaaaagtgg-----cc
SEQ ID NO:28	302 ----aaact-----acctgtgaa-----gg-----a-
SEQ ID NO:29	310 ggtgaaaccacctgcgacggcaaaaagaaaa-----tgtatgaatac-
SEQ ID NO:25	338 ----acggcaaaagct----ccggtcatcg-tcttcacacagccgcagaa
SEQ ID NO:27	343 atgaaagacaagatg----ccggttattt-tcctggctcatccccaggtc
SEQ ID NO:28	319 ----aagaagaagat----gtttgagttgatggagagattggtgccaatg
SEQ ID NO:29	352 ----atggcggagtttaagcctgttcatg-tgatgcaattgcccacagc
SEQ ID NO:25	379 cgtaaga-tccgcccggc-----tgctgatttctctcaaag-ct
SEQ ID NO:27	388 cgtcagaatgccgcggc-----aagc-agttcacctatg-at
SEQ ID NO:28	361 catataa-tgcacctcccacacatgaaagatgaagattctttgaaaatct
SEQ ID NO:29	397 gttaagg-acgatgcctc-----gcgtgcgttatggaaag-cc
SEQ ID NO:25	415 gaat--acgaacatgtc---cgt-----acgg--aattgg---gacg
SEQ ID NO:27	424 gcct--acagcgaagt---ga-----aaggccatctgg---aaga
SEQ ID NO:28	410 ggattaagaagttgaaaagcta-----aaag--aattggttgagaaa
SEQ ID NO:29	433 ga-----gatgctgcg---cttgcaaaaaacgg--tagaag---aacg

SUBSTITUTE SHEET (RULE 26)

28/105

SEQ ID NO:25	447	tatcctcaacgtaaaa--atctccgacctggctatccaggaagctatcaa
SEQ ID NO:27	456	aatctgcgccatgaa--atcaccaatgatgccatcctggatgccatcaa
SEQ ID NO:28	451	gagactggaaaataaaataacagaggaaaagttaaagagacagttgataa
SEQ ID NO:29	468	ttttgggcacg---ag--attagcgaagatgctctgcgcgatgccattgc
SEQ ID NO:25	495	agtatataacgaaaaccgtcaggttatgcgtgaattct-----gcg
SEQ ID NO:27	504	agtgtaacaagaagccgtgctgccgcgcgcgaattct-----gca
SEQ ID NO:28	501	agtaataaaagtta---gggagttgtttataaaactct-----atg
SEQ ID NO:29	513	gctgaaaaaccgcgaacgtcgcgcactggctaatttttatcatcttgggc
SEQ ID NO:25	536	acgtagctgctcag-----taccgcgagatcttcaactccgataaa--acg
SEQ ID NO:27	545	aactggc---caacg-----aacatcctgatctgatcccggtctccgtacg
SEQ ID NO:28	539	a-attgaggaagaa-----taaac-cag-----ctccaattaa---ggg
SEQ ID NO:29	563	agttaaatcctccggcgcttagcggcag---cgacattctgaaagt---ggt
SEQ ID NO:25	579	tcatgacgtcatc-----aaag---cccgctgg-----ttca
SEQ ID NO:27	588	ggccaccgtactg-----cgtg-----ccgcttac-----ttca
SEQ ID NO:28	573	tttagatgtttta-----aaattattccagtttgctatttat
SEQ ID NO:29	609	ttacggcgcaaccttccggttcgataaaag---aggcgttg-----atca
SEQ ID NO:25	608	tggacaaagctgaacacaccgctttggtcgcgaactcatcgacgtgctc
SEQ ID NO:27	617	tgctgaaggatgaatacacccgaaaagctggaagaactgaacaagg-----
SEQ ID NO:28	611	tggatattgatgacacaatagggaatttagaggatttaattgaggagttta
SEQ ID NO:29	650	atgaactggatgcaatgaccgc-----ccgcg---ttcgtcagcagtgagg
SEQ ID NO:25	658	aagaa-----ag---aaccggtacagccgtggaat-----ggcaaaaaa
SEQ ID NO:27	662	aactg-----gc---agctgctcctgccggcaagttcagcggccacaaa
SEQ ID NO:28	661	gaggagagagttaa---aaaaggagaaggttatgaa-----ggaaagaga
SEQ ID NO:29	692	aagaa-----ggccagcgactggaccgcgctccg-----cgcatttta
SEQ ID NO:25	694	gtcatcctctccggt-----atcatggcagaaccggatgaattcct---
SEQ ID NO:27	703	gtgggtgtttccggc-----atcatctacaacacgcccggcatcct---
SEQ ID NO:28	703	attttaataactggctgtccaatgggttgctggaacataagattgt---
SEQ ID NO:29	730	atcacccggtgcccgc-----attggcggcgagcagaaaaagtggtgcg
SEQ ID NO:25	735	cgatatcttcagcgaatt-caacatcgctgctgctgctgacgacctc-gc
SEQ ID NO:27	744	gaaagccatggatgacaa-caaactggccattgctgctgatgactgc-gc
SEQ ID NO:28	750	tgaaattattgagggaagt-tggaggagtagttgttggtgaaagaaagctgc
SEQ ID NO:29	774	cgcgat-tgaagagaatggcggctgggttgctggttatgaaaactgc-ac
SEQ ID NO:25	783	tcagga-atcccgcagttccgtacagacgtaccgtccggcatcgatccc
SEQ ID NO:27	792	ttatga-aagccgcagctttgccgtggatgctccggaagatctgga---c
SEQ ID NO:28	799	actgga-a-----caagattctttgaaaactttgttgagg---gctatagc
SEQ ID NO:29	822	cggggcgaaagcgaccgagcaatgc-gtggcagaaaacggg---cgatgctc
SEQ ID NO:25	832	ctcgaacagctcgctcag-----cagtggtg-----caggacttcgat-g
SEQ ID NO:27	838	aacggactgcatgctctggctgtacagttctccaacagaagaacgat-g
SEQ ID NO:28	841	gtagaggacattgcaaa-----aaga-tacttt-a
SEQ ID NO:29	868	tacgacgcgctggcggat-----aaatat-----ctgg---cgattg
SEQ ID NO:25	869	----gctgccgctcgctttgaa-----cgaagacaaaccgcg-tggccag
SEQ ID NO:27	887	ttctgtgtacgatcc---tgaatttgccaagaataccggttctgaacac
SEQ ID NO:28	869	----aaatcccatgtgcttgta-----gatttaaaaacgat-gagagag
SEQ ID NO:29	902	----gctgctc-ctgtgtttcgc---cga--acgatcagcg-cctgaaa
SEQ ID NO:25	910	atgctcatcgaca-----tgactaagaaatacaatgctgacgcgcgtcgtc
SEQ ID NO:27	934	gttggca---atc-----tggtaaaagaaagcggcgagaaaggactgatc
SEQ ID NO:28	908	ttgaaaatataaagagattggttaaagagttggacgtcgatggagttgtt
SEQ ID NO:29	940	atgctcagccaga-----tggtggaggaaatcacggtogatggcgtagtt

29/105

SEQ ID NO:25 955 atctgcatgatgcgtttctgcatcctgaagaattcgactatc---cgat
SEQ ID NO:27 976 gtgttcgatgatgcagttctgcatccggaagaaatggaptatc---ctga
SEQ ID NO:28 958 tattacactttgcagtattgccatacatttaacatagaggggag---ctaa
SEQ ID NO:29 985 gatgtgattttgcaggcgtgccatacctacgcggtggaatcgctggcgat

SEQ ID NO:25 1002 ttacaaaccggaatttgaagctgctgg----cgttcgttacacggctctc
SEQ ID NO:27 1023 tctgaagaaggctctggtatgccacca----cattcctcatgtgaagatt
SEQ ID NO:28 1005 ggtagaggaggcattaaaagaggaggg----cattc-----caattata
SEQ ID NO:29 1035 t-----aaacgtcatgtgcgccagcagcacaacattccttatatcgctatt

SEQ ID NO:25 1048 gacctcgacatcgaatctccgtccctcgaa-----cagctccgcaccccg
SEQ ID NO:27 1069 ggtgtggaccagatgacccgggactttggt-----caggcccagaccgc
SEQ ID NO:28 1045 agaattgaaactgactattctgaaagtgatagagagcagttaaaaacaag
SEQ ID NO:29 1081 gaaacagactactccacctcggatgtcggg-----cagctcagtagcccg

SEQ ID NO:25 1092 tatccaggctttctcggaatcctctaa
SEQ ID NO:27 1113 tctggaagctttcgagaaagcctgtaa
SEQ ID NO:28 1095 gttggaggcatttattgagatgatttaa
SEQ ID NO:29 1125 tgtcgcggcctttattgagatgctgtaa

SUBSTITUTE SHEET (RULE 26)

30/105

Figure 21

SEQ ID NO:26	1 msqidelisklqevsnhpqk---tvlnykkqgkglvgmmpyyapeei vya
SEQ ID NO:30	1 -maisalieefqkvsaspt---mlakykagkkaigclpyyvpeel vya
SEQ ID NO:31	1 mmkl-kaieklmqkf asrke---qlykqkeegrkvfgmfca yvpieila
SEQ ID NO:32	1 mslvtdlpaifdqfsearqtgfltvmdlker giplvgtyctfmpqeipma
SEQ ID NO:26	48 agylpvgmfgsqnpqisaartylppfacslmqadmelqlngt---ydc--
SEQ ID NO:30	47 agmvpmgvwcngkqevrskeycasfyctiaqqslemlldgt---ldg--
SEQ ID NO:31	47 anaipvglcggkndtipiaeedlprnlcp likssygfkkaktcp yfea--
SEQ ID NO:32	51 agavvvsclcstsdetieeae kdlprnlcp likss---ygf gkt---dkc py
SEQ ID NO:26	93 ---ldavifsvpcdtlrcmsqkwh---gkapv ivftqpqrki rpavdf
SEQ ID NO:30	92 ---ldgiitpvlcdtlrpsqnfkvamkdkmpviflahpqvrqnaagkqf
SEQ ID NO:31	95 ---sdvigetttceggkkmfelme---rlvpmhimhlp-hmkdedslki
SEQ ID NO:32	96 fyfsdlvvgettcdgkkkmyeyma---efkpvhvmqlpnsvkddasral
SEQ ID NO:26	136 lkaeyehvrtelgrilnvkisdlaiqealkvynenrqvmrefcdvaagyp
SEQ ID NO:30	139 tydaysevkghleeicqheitndaildaikvynksraarrefcklanehp
SEQ ID NO:31	137 wikeveklkelveketgnkiteekketvdkvkvrelfyklyelrknkp
SEQ ID NO:32	142 wkaemrlrktveerfgheisedalrdaialkmrerralanfyhlqqlnp
SEQ ID NO:26	186 giftpikrhdivk---arwf---mdkaehtalvrelidavkk--epvqp
SEQ ID NO:30	189 dlipasvratvtr---aayf---mlkdeytekleelnkelaa--apagk
SEQ ID NO:31	187 ---apikgldvik---lfqfaylliddttigiledlieeleervkkgqg
SEQ ID NO:32	192 ---palgsdilkvvygatfr---fdk---ealinel-damta--rvrqq
SEQ ID NO:26	227 wn-gkk-----vilsg--imaepdefldifsefniavvaddlaqesrqf
SEQ ID NO:30	230 fd-ghk-----vvvsg--iiyntpgilkamddnklaiaddcayesrsf
SEQ ID NO:31	230 ye-gkr-----ilitgcpmvagnnkiveiieevggvvvgeesctgtrff
SEQ ID NO:32	230 weegqrlqprlilitgcpiggaaekvvraieenggwvvygencgakat
SEQ ID NO:26	268 rtdvpsgidp-leqlaqwqdfdgcpalned---kprgqmlidmtkkyn
SEQ ID NO:30	271 avdapedldnglhalavqfskqkndvll ydpefakntrsehvgnlvkesg
SEQ ID NO:31	273 enfv-egys--vediakyfkip-cacr fknnd---e-rvenikrlvkeld
SEQ ID NO:32	280 eqcvaetgdv-ydaladkylai-gcscvspnd---q-rlkmlsqmveeyq
SEQ ID NO:26	314 adavvicmmrfcdpeefdypiykpef-eaagvrytvldldiespsleqlr
SEQ ID NO:30	321 aeglivfmnqfcdpeemeypdllkal-dahhiphvkigvdqmrdrfggaq
SEQ ID NO:31	315 vdgvytytlqychtfniegakveeal-keegipiirietdysesdreqlk
SEQ ID NO:32	324 vdgvvdivlqachtyaveslaikrhvrqqhni pyiaietdystsdvgqls
SEQ ID NO:26	363 triqafseil
SEQ ID NO:30	370 taleafaesl
SEQ ID NO:31	364 trleafiemi
SEQ ID NO:32	374 trvaafiemi

31/105

Figure 22

1 CGAOGGCCCG GGCTGGTATC ATTCTAGTCA GTAATTCACC TTTGGAAAAT TTTACACAAAG
61 GCAGTACGAC AGAAGCGTCG ATACATTCCA TTTAGCAGGA GGAAGTTACG GTAATGAGAA
121 AAGTAGAAAT CATTACAGCT GAACAAGCAG CTCAGCTCGT AAAAGACAAC GACACGATTA
181 CGTCTATCGG CTTTGTACAG AGCGCCATC CGGAAGCACT GACCAAAGCT TTGGAAAAAC
241 GGTTCCTGGA CACGAACACC CCGCAGAACT TGACCTACAT CTATGCAGGC TCTCAGGGCA
301 AACGCGATGG CCGTGCCGCT GAACATCTGG CACACACAGG CCTTTTGGAA CGCGCCATCA
361 TCGGTCAC TG CAGACTGTA CCGGCTATCG GTAAACTGGC TGTCGAAAAC AAGATTGAAG
421 CTTACAATT CTGCGAGGAC ACCTTGGTCC ACTGGTTCOG CGCCTTGGCA GGTCTAAGC
481 TCGGGTCTT CACCGACATC GGTCTGAAA CTTTCTCGA TCCCCGTCAG CTCGGCGGCA
541 AGCTCAATGA CGTAACCAA GAAGACCTCG TCAAAGTATG CGAAGTCGAT GGTCTGAAC
601 AGCTTTTCTA CCCGACCTTC CCGGTCAAAG TAGCTTTCCT CCGCGGTACG TATGCTGATG
661 AATCCGCGCA TATCACCATG GACGAAGAAA TCGGGCCCTT CGAAGCACT TCCGTAGCC
721 AGGCGGTTCA CAACTGTGGC GGTAAAGTCG TCGTCCAGGT CAAAGACGTC GTCGCTCAGC
781 GCAGCTCGA CCCGCGCATG GTCAAGATCC CTGGCATCTA TGTCGACTAC GTCGTCTAG
841 CAGCTCCGGA AGACCATCAG CAGACGTATG ACTGCGAATA CGATCCGTCC CTGACCGGTG
901 AACATCGTGC TCCTGAAGGC GCTACCGATG CAGCTCTCCC CATGAGCGCT AAGAAAATCA
961 TCGGCGCGCG CGGCGCTTTG GAATTGACTG AAAACGCTGT CGTCAACCTC GCGGTCTGCG
1021 CTCCGGAATA CGTTGCTTCT GTTGCCGGTG AAGAAGGTAT CGCCGATACC ATTACCTGA
1081 CCGTCGAAGG TGGCGCCATC GGTGGCGTAC CGCAGGGGCG TGCCGCTTC GGTCTGTC
1141 GCAATGCGGA TGCCATCATC GACCAACCT ATCAGTTGCA CTTCTACGAT GCGCGCGGTC
1201 TGGACATCGC TTACCTCGGC CTGGCCAGT GCGATGGCTC GGGCAACATC AACGTCAACA
1261 AGTTCCGTAC TAACGTTGCG GGTGCGGCG GTTTCGCCAA CATTTCOCAG CAGACACCGA
1321 ATGTTTACTT CTGCGGCAOC TTCAOGGCTG GCGGCTTGA AATCGCTGTC GAAGAOCGCA
1381 AAGTCAAGAT CCTCCAGGAA GGCAGAGCCA AGAAGTTCAT CAAAGCTGTC GACCAGATCA
1441 CTTTCAACGG TTCTATGCA GCCCGCAACG GCAATCACGT TCTCTACATC ACAGAACGCT
1501 GCGTATTGA ACTGACCAA GAAGGCTTGA AACTCATCGA AGTCGCCAGG GGCATCGATA
1561 TTGAAAAGA TATCTCGCT CACATGGACT TCAAGCCGAT CATTGATAAT CCGAAACTCA
1621 TGGATGCGCG CCTCTOCAG GACGGTOCCA TGGGACTGAA AAAATTAATC TCTGCTGTAA
1681 AGGAGACTTT ACTATGAAC CAATGAGACT ACATCACGTA GGCATTGTCC TGCCGACCTT
1741 AGAAAAAGCC CATGAATCA TGCAGAATAA TGGACTGAA ATCGACTATG CCGGCTATGT
1801 CGATGCTTAC CAGGCTGATC TCATTTCAC TAAGTTTGGT AATTTGCCA GCCCGATTGA
1861 AATGATTATC CCGCACTCG GTGTGCTTAC CCAATTCAAT GGTGGCGCG GCGGCATTGC
1921 CCACATCGCC TTCGAAGTGG ACGATGTGCA AGCTGTCCG CAGGAATAGG AAGCAGATTG
1981 TCCGGGATGC ATGTTAGAAA AGAAGCTGT CCAGGCTAGC GACGACATA TCGTCAACTT
2041 CGCGCGCGCG ACAACCAAOC AGGGTATCCT CGTTGAATAT GTTCAGAGA CAGCACTAT
2101 CACCGGCGCG GCGGAAAATC CTTTCGTTAA GAATCTCGGC CCGGAAAAG GGAAGCTCAA
2161 CGAACATGG CATCCCATGC GCCTGCAACA TATCGGCATC GTCTTGCGA CCTTGGAAAA
2221 GGCCCATGAA TTCATCAAGA CCAATGGTCT GGAAGTGGAT TATTCGGTT TCGTCGAGCG
2281 CTACCATGCG GATCTCATTT TCACTAAAAA AGGTGAAAC AGTACGCTA TCGAATTCTAT
2341 TATTCCCGGT GAAGGGGTCC TCAAAGATT CAATCATGGC AGGGGAGGTA TCGCTCATAT
2401 CGCCTTTGAA GTGGATGATG TCGAAAAGGT ACGTCAGATT ATGGAAGCC AGAAGCCTGG
2461 TTGCATGCTC GAAAAGAAAG CCGTCCGGGG AACGACGAT ATCATCGTCA ACTTCCGCGG
2521 TCCAGCAGC GACGCGGCA TCCTCGTGA ATATGTCCAG ACCGTAGCTC CCATCAATCG
2581 CAGCAATCC AACCTTTTA ATGATTGATT TTTTATAAAG AAAGGTGAAA ACTGTGTATA
2641 CTCTCGGAAT CGACGTTGCT TCTTCTTCT CCAAGGCAGT CATCTGGAA GATGGCAGA
2701 AGATCGTCCG CCATGCGCTC GTTGAATCG GCACCGGTT GACCGGTCC GAACGCGTCC
2761 TGGACGAAGT CTTCAAGAT ACCAATCTAA AAATTGAAGA CATGGCGAAC ATCATCGCCA
2821 CAGGCTATGG CCGTTTCAAT GTCGACTGCG CCAAAGGCGA AGTCAGCGAA ATCAGCTGCC
2881 ATGCCAAAGG GGCCTCTTT GAATGCCCG GTACGACGAC CATCTCGAT ATCGGCGGTC
2941 AGGACGTCAA GTCCATCAA TTGAATGGCC AGGGCTGGT CATGCACTT GCCATGAACG
3001 ACAATGCGC CGCTGGTAGG GGCGGTTTC TCGACGTCAT GTCGAAGGTA CTGGAATCC
3061 CCATGCTGTA AATGGGGGAC TGGTACTTCA AATCGAAGCA TCCCGCTGCC GTCAGCGTA
3121 OCTGCAAGGT TTTGCTGAA TCGGAAGTCA TTTCCCTTCT TTCCAAGAA GTCCCGAAG
3181 AAGATATCGT AGCCGGTGT CATCAGTCCA TCGCGGCCAA AGCCGCGCT CTCCTGGGCG
3241 GCGTCGGTGT CCGTGAAGAC CTGACCATGA CCGGCGGTGG CTCCCGCGAT CCCGGCGTGG
3301 TCGATGCCGT ATCGAAAGAA TTAGGTATTC CTGTACAGAT CGCTCTGCAT CCCAAGCGG
3361 TGGTGCTCT CCGAGCTGCT TTGATTGCTT ATGATAAAAT CAAGAAATAA GTCAAAGGAG

SUBSTITUTE SHEET (RULE 26)

32/105

3421 AGAACAAAT CATGAGTGAA GAAAAACAG TAGATATTGA AAGCATGAGC TCCAAGGAAG
3481 CCCTTGGTTA CTCTTGCCG AAGTCGATG AAGACGCAAG TAAAGCGAAA AAAGAAGGCC
3541 GCCTCGTTTG CTGGTCCGCT TCTGTCGCTC CTCCGGAATT CTGCACGGCT ATGGACATCG
3601 CCATCGTCTA TCCGAAACT CACGCAGCTG GTATCGGTGC CCGTCACGGT GCTCCGGCCA
3661 TGCTCGAAGT TGCTGAAAC AAAGGTTACA ACCAGGACAT CTGTTCTTAC TGCCCGCTCA
3721 ACATGGGCTA CATGGAACCT CTCAAACAGC AGGCTCTGAC AGGCGAAAGC CCGGAAGTCC
3781 TCAAAAACCTC CCGGCTTCT CCGATTCCCC TTCCGGATGT TGTCTCACT GCTTCAACA
3841 TCTGCAATAC CTGCTCAAA TGGTATGAAA ACTTGGCTAA AGAATTGAAC GTACCTCTCA
3901 TCAACATCGA CGTACCGTTC AACCATGAAT TCCTGTGTAC GAAACACGCT AAACAGTACA
3961 TCGTCGGGGA ATTCAACAT GCTATCAAAC AGCTCGAAGA CCTTTGCGGC CGTCCCTTCG
4021 ACTATGACAA ATTCTTGAA GTACAGAAAC AGACACAGCG CTCCATCGCT GCTTGAACA
4081 AAATCGCTAC GTACTTCCAG TACAAACCGT CGCCGCTCAA CGGCTTGCAC CTCTTCAACT
4141 ACATGGGCTC CGCGTTGCT GCCCGCTCCT TGAACTACTC GGAATCACG TTCAACAAAT
4201 TCCTCAAAGA ATTGGACGAA AAAGTAGCTA ATAAGAAATG GGCTTTCCGT GAAAACGAAA
4261 AATCCCGTGT TACTTGGGAA GGTATCGCTG TCTGGATCGC TCTCGGCAC ACCTTCAAG
4321 AACTCAAAGG TCAGGGGCTC CTCATGACTG GTTCCGCTTA TCCTGGCATG TGGGACGTTT
4381 CCTACGAACC GGGCGACCTC GAATCCATGG CAGAAGCTTA TTCCCGTACA TACATCAACT
4441 GCTGCCTCGA ACAGCGCGGT GCTGTTCTTG AAAAGTTGT CCGCGATGGC AAATGCGAGC
4501 GCTTGATCAT GCACCAAGAC GGTCTCTGCA AGAACATGAG CCTCTCAAC AACGAAGGCG
4561 GCCAGCGCAT CCAGAAGAAC CTCGGCGTAC CGTACGTCAT CTTGACGGC GACGAGACCG
4621 ATGCTCGTAA CTCTCGGAA GCACAGTTCC ATACCCGCGT AGAAGCTTTG GCAGAAATGA
4681 TGGCAGACAA AAAAGCCAAAT GAAGGAGGAA ACCACTAATG AGTCAGATCG ACGAACTTAT
4741 CAGCAAATTA CAGGAAGTAT CCAACCATCC CCAGAAAGCG GTTTTGAATT ATAAAAACA
4801 GGGAAGAGG CTCTAGGCA TGATGCCCTA CTACGCTCCG GAAGAAATCG TATATGCTGC
4861 AGGCTACCTC CCGTAGGCA TGTTCGGTTC CCAGAACCGG CAGATCTCCG CAGCTCGTAC
4921 GTACCTTCTC CCGTTCGCTT GCTCCTTGTAT GCAGGCTGAC ATGGAACTCC AGCTCAACGG
4981 CACCTATGAC TGCCCTGACG CTGTTATCTT CTCGTTCTCT TCGGACACTC TCCGCTGCAT
5041 GAGCCAGAAA TGGCAGGCA AAGCTCCGGT CATCGTCTTC ACACAGCCGC AGAACCGTAA
5101 GATCCGCCCG GCTGTGATTT TCCTCAAAGC TGAATACGAA CATGTCCGTA CCGAATTGGG
5161 ACGTATCCTC AACGTAAAAA TCTCCGACCT GGCTATCCAG GAAGCTATCA AAGTATATAA
5221 CGAAACCGT CAGGTTATGC GTGAATTCTG CGACGTAGCT GCTCAGTACC CGCAGATCTT
5281 CACTCCGATA AAACGTCATG ACGTCATCAA AGCCCGCTGG TTCATGGACA AAGCTGAACA
5341 CACCGCTTGG GTCCCGCAAC TCATCGACGC TGTCAAGAAA GAACCGGTAC AGCCGTGGAA
5401 TGGCAAAAAA GTCATCCTCT CCGGTATCAT GGCAGAACCG GATGAATTCC TCGATATCTT
5461 CAGCGAATTC AACATCGCTG TCGTCGCTGA CGAAGCTGCT CAGGAATCC GCCAGTTCG
5521 TACAGACGTA CCGTCCGCA TCATCCCTC CGAACAGCTC GCTCAGCAGT GGCAGGACTT
5581 CGATGGCTGC CCGCTCGCTT TGAACGAAGA CAAACCGGT GGCCAGATGC TCATCGACAT
5641 GACTAAGAAA TACATGCTG ACGCCGTCGT CATCTGCATG ATGCGTTTCT GCGATCTGA
5701 AGAATTGAC TATCCGATTT ACAAACCGGA ATTTGAAGCT GCTGGCGTTC GTTACACGGT
5761 CCTCGACCTC GACATCGAAT CTCGTCCTC CGAACAGCTC CGCAACCGTA TCCAGGCTTT
5821 CTCGGAATTC CTCTAAGAAT CGCTGAATC ATCAAAATC TGGGCGGGAC TCCGAAGGT
5881 GCTGCTACA TGATACATTG OCTGTTTTCA GGCAGACAGA TTTGCAGCTT GCGGCCOCCA
5941 TTGTACGGGC TGCAAGCTGT CAATGATGCT TTAAAGACGG CTCTGCGGTT TTTAAATAAA
6001 AACATAAAAC CATATATAAT CTATTAGGAG GAACTCAAT CATGGAATTC AAACCTTCTG
6061 AATTACAGCA AGATATCGCA AATCTCGCAA AAGATTTGCG AGAAAAAAA TTAGCTOCCA
6121 CTGTCAAAGA GCGTGACGAA AAAGAAGTTT TCGATCGTGC TATCCTTGAC GAAGTGGGTA
6181 CTCTCGGCT TCTCGGTATT CCTGGGAAG AAGAAACCGG CCGCGTAGGC GCTGACTTCC
6241 TCAGCCTCGC AGTTGCTTGC GAAGAAGTAG CTAAAGTTAC CAGCCCGGGC CGTGC (SEQ

ID NO:33)

33/105

Figure 23

ATGAAACCAATGAGACTACATCAGTAGGCATTGTCCTGCCGACCTTAGAAAAAGCCCAT
GAATTCATGCAGATAATGGACTTGAATCGACTATGCCGGCTATGTCGATGCTTACCAG
GCTGATCTCATTTTCACTAAGTTTGGTGAATTTGCCAGCCCGATTGAAATGATTATCCCG
CACTCCGGTGTGCTTACCCAATTCAATGGTGGCCGCGGGCGGCATTGCCACATCGCCTTC
GAAGTGGACGATGTCGAAGCTGTCCGCCAGGAAATGGAAGCAGATTGTCCGGGATGCATG
TTAGAAAAGAAAGCTGTCCAGGGTACGGACGACATTATCGTCAACTTCCGCCGCCCGACA
ACCAACCAGGGTATCCTCGTTGAATATGTTTCAGACGACAGCACCTATCACCGGCCGCGGC
GAAAATCCTTTTCGTTAAGAATCTCGGCCCGGAAAAAGGGAAGCTCAACGAAACATGGCAT
CCCATGGCCTGCACCATATCGGCATCGTCTTGCCGACCTTGAAAAGGCCCATGAATTC
ATCAAGACCAATGGTCTGGAAGTGGATTATTCCGGTTTCGTGACGCCTACCATGCGGAT
CTCATTTTCACTAAAAAGGTGAAAACAGTACGCCTATCGAATTCATTATCCCCGTGAA
GGGGTCCTCAAAGATTTCAATCATGGCAGGGGAGGTATCGCTCATATCGCCTTTGAAGTG
GATGATGTCGAAAAGGTACGTCAGATTATGGAAAGCCAGAAGCCTGGTTCATGCTCGAA
AAGAAAGCCGTCCGGGGAACGGACGATATCATCGTCAACTTCCGCCGTCCAGCACGGAC
GCCGGCATCCTCGTGAATATGTCCAGACCGTAGCTCCCATCAATCGCAGCAATCCCAAC
CCTTTAATGATTGA (SEQ ID NO:34)

SUBSTITUTE SHEET (RULE 26)

34/105

Figure 24

MKPMRLHHVGIVLPTLEKAHEFMQNNGLEIDYAGYVDAYQADLFTKFGFASPIEMIIP
HSGVLTQFNNGRGGIAHIAFEVDDVEAVRQEMEADCPGCMLEKKAVOGTDDIIVNFRPT
TNQGILVEYVQTTAPITGRGENPFVKNLGPEKGKLNETHWPMRLHHIGIVLPTLEKAHEF
IKTNGLEVYSGFVDAYHADLFTKKGENSTPIEFIIIPREGVLKDFNHGRGGIAHIAFEV
DDVEKVRQIMESQKPGCMLEKKAVRGTDIIIVNFRPSTDAGILVEYVQTVAPINRSNP
PFND (SEQ ID NO:35)

35/105

Figure 25

ATGGAATTCAAACCTTCTGAATTACAGCAAGATATCGCAAATCTCGCAAAAGATTTGCA
GAAAAAATTAGCTCCCACTGTCAAAGAGCGTGACGAAAAAGAAGTTTTCGATCGTGCT
ATCCTTGACGAAGTGGGTACTCTCGGCCTTCTCGGTATTCCCTGGGAAGAAGAAAACGGC
GGCGTAGGCGCTGACTTCCTCAGCCTCGCAGTTGCTTGCGAAGAAGTAGCTAAAGTTACE
AGCCCGGGCCGTCG (SEQ ID NO: 36)

SUBSTITUTE SHEET (RULE 26)

36/105

Figure 26

MEFKLSELQQDIANLAKDFAEKKLAPTVMKERDEKEVFDRAILDEVGTIGLLGIPWEEENG
GVGADFLSLAVACEEVAKVTSPGR (SEQ ID NO:37)

37/105

Figure 27

```

1  GTGAGCACAC ACTTGATAGC TGATGCCGTC AATGATCAGT TGTTCGTCTA TAGCAGGCTG
61  AAAGGACATG GGTTTGGTCA CAGTCTGAGC AGTTGCAGGC AGTCAAACAC GTTCGTAACT
121  ACGCTGTAGA TGATATAAGC AGTATACCAT CTGCTACGC TCTCGTTGAT CAGGTTGAAT
181  GCTTTGAGGA AGGTCAGGCG AATAGCCATG CCTCTTGTTT CCAGAACATG GCATGGGGAT
241  GGATCGACGG TACCCCTGTCG GATGCATGCT ATGCGTGCCA TTCATATCAT CAACCAGAAAT
301  TTGATCTTGA ACTACACAGC AATCTGCGC GTTATGCAAG TGTCTTCGGT CAGATGGTGA
361  ACAATTCTCA ATTGTTGAGG TCTTGACGAA TTGCGTTATA CACTGTAGGC TATAGTATGC
421  ACCCCTTGTT ATCTATATCA CAACCGGTCT ATTAGCATTT GCGTCAAGGA GGATGGTCTGA
481  TGATCGACAC TGGGCCOCTT GCCCCACCAC GGGCGOCCCG CTCTAATCCG ATTCGGGATC
541  GAGTTGATTG GGAAGCTCAG CGCGCTGCTG CGCTGCCAGA TCCCGGTGOC TTTCTATGGCG
601  CGATTGCCCG GACAGTTATC CACTGGTAGC ACCACAACA CCATTGCTGG ATTCGCTTCA
661  ACGAGTCTAG TCAGOGTTGG GAAGGGCTGG ATGCCGCTAC CGGTGCCOCT GTAACGGTAG
721  ACTATCCCGC CGATTATCAG CCCTGGCAAC AGGCGTTTGA TGATAGTGAA GCGCCGTTTT
781  ACCGCTGGTT TAGTGGTGGG TTGACAAATG CCTGCTTTAA TGAAGTAGAC CGGCATGTCA
841  TGATGGGCTA TGGCGACGAG GTGGCTACT ACTTTGAAGG CACTGCTGG ATTCACTCGC
901  TCAACAATGG TCGTGGTGGT CCGGTTGTCC AGGAGACAAT CACGOGGCGG CGCCTGTGG
961  TGGAGGTGGT GAAGGCTGCG CAGGTGTGTC GTGATCTGGG CCTGAAGAAG GGTGATCGGA
1021  TTGCTCTGAA TATGCCGAAT ATTATGCCGC AGATTTATTA TACGGAAGCG GCAAAACGAC
1081  TGGGTATTCT GTACACGCCG GTCTTCGGTG GCTTCTCGGA CAAGACTCT TCCGACCGTA
1141  TTCACATGCG CGGTGCACGA GTGGTGATTA CCTCTGATGG TGGTAOCCG AACCGCGCAG
1201  TGGTGCCCTA CAAAGAAGCG TATACCGATC AGGCGCTCGA TAAGTATATT CCGGTTGAGA
1261  CGGCGCAGGC GATTGTTGCG CAGACCTGCG CCAOCTTGCC CCTGACTGAG TOGCAGCGOC
1321  AGACGATCAT CACCGAAGTG GAGGCGCAC TGGCCGGTGA GATTACGGTT GAGCGCTCGG
1381  ACGTGATGCG TGGGGTTGGT TCTGCCCTCG CAAAGCTCG CGATCTTGAT GCAAGCGTGC
1441  AGGCAAAGGT GCGTACAGTA CTGGCGCAGG CGCTGGTCGA GTCCGCGCGG CGGGTTGAAG
1501  CTGTGGTGGT TGTGCGTCAT ACCGGTCAGG AGATTTGTG GAAOGAGGGG CGAGATCGCT
1561  GGAGTCAAGA CTTGCTGGAT GCTGCCCTGG CGAAGATTCT GGCCAATCGG CGTGCTGCCG
1621  GCTTTGATGT GCACAGTGAG AATGATCTGC TCAATCTCCC CGATGACCAG CTTATCCGTG
1681  CGCTCTACGC CAGTATTCCC TGTGAACCGG TTGATGCTGA ATATCCGATG TTTATCATT
1741  ACACATCGGG TAGCACCGG AAGCCCAAGG GTGTGATCCA CGTTCAOGCG GGTATGTGCG
1801  CCGGTGTGGT GCACACCTTG CCGGTCAGTT TTGACGCCGA GCGGGGTGAT ACGATATATG
1861  TGATGCGCGA TCCGGGCTGG ATCACCCTGC AGAGCTATAT GCTCACAGCC ACAAATGGCG
1921  GTCCGCTGAC CGGGGTGATT GCCGAGGGAT CACCGCTCTT CCGCTCAGCC GGGGGTTATG
1981  CCAGCATCAT CGAGCGCTAT GGGGTGCGA TCTTTAAGGC GGGTGTGACC TTCTCAAGA
2041  CAGTGATGTC CAATCCGCG AATGTTGAAG ATGTGCGACT CTATGATATG CACTCGCTGC
2101  GGGTTGCAAC CTTCTGCGCC GAGCCGGTCA GTCCGCGGGT GCAGCAGTTT GGTATGCGA
2161  TCATGACCCC GCAGTATATC AATTCGTACT GGGCGACCGA GCACGGTGGA ATTGTCTGGA
2221  CGCATTCTA CGGTAATCAG GACTTCCCGC TTCGTCCCGA TGCCCATACC TATCCCTTGC
2281  CCTGGGTGAT GGGTGATGTC TGGGTGGCCG AACTGATGA GAGCGGACG ACGCGCTATC
2341  GGGTGGCTGA TTTCTGATGAG AAGGGCGAGA TTGTGATTAC GCGCCCGTAT CCTAOCCTGA
2401  CCGCACACT CTGGGGTGAT GTGCCGGTT TCGAGGCGTA CCTGCGGGT GAGATTCCGC
2461  TCGGGGCTG GAAGGGTGAT GCCGAGCGTT TCGTCAAGAC CTACTGGCGA CGTGGGCCAA
2521  ACGGTGAATG GGGCTATATC CAGGGTGATT TTGCCATCAA GTACCCCGAT GGTAGCTTCA
2581  CGCTCCACGG ACGCCCTGAC GATGTGATCA ATGTGTGGG CCACCGTATG GGCACCGAGG
2641  AGATTGAGGG TGCCATTTTG CGTGACCGCC AGATCACGCC CACTCGCCC GTCCGTTAAT
2701  GTATTGTGGT CCGTGCGCGG CACCGTGAGA AGGGTCTGAC CCGGTTGCC TTCATTCAAC
2761  CTGCGCCTGG CCGTCATCTG ACCGCGCGCG ACCGGCGCGG TCTCGATGAG CTGGTGCGTA
2821  CCGAGAAGGG GCGGTCAGT GTCCAGAGG ATTACATGA GGTCAGTGCC TTTCCCGAAA
2881  CCGCAGCGG GAAGTATATG CCGCGCTTTT TCGCAATAT GATGCTGAT GAACCACTGG
2941  GTGATACGAC GACGTTGCGC AATCTGAGG TGCTCGAAGA GATTGACGCC AAGATCGCTG
3001  AGTGGAACG CGTCAAGCT ATGGCCGAAG AGCAGCAGAT CATCGAACGC TATCGCTACT
3061  TCCGGATCGA GTATCAACCA CCAACGGCCA GTGCGGGTAA ACTCGCGGTA GTGACGGTGA
3121  CAAATCCGCC GGTGAACGCA CTGAATGAGC GTGCGCTCGA TGAGTTGAAC ACAATTGTTG
3181  ACCACCTGGC CCGTCTGAG GATGTTGCCG CAATTGTCTT CACCGGACAG GCGCCAGGA
3241  GTTTGTGCGC CGGCGCTGAT ATTCGCCAGT TGCTCGAAGA GATTCATACG GTTGAAGAGG
3301  CAATGGCCCT GCCGAATAAC GCGCATCTTG CTTTCCGCAA GATTGAGCGT ATGAATAAGC
3361  CGTGATGCG GCGATCAAC GGTGNGCGC TCGGTGGTGG TCTGGAATTC GOCATGGCCT

```

SUBSTITUTE SHEET (RULE 26)

38/105

3421 GCCATTACCG GGTGCGGAT GTCTATGCG AATTCGGTCA GCCAGAGATT AATCTGCGCT
3481 TGCTACCTGG TTATGGTGGC ACGCAGCGCT TGCCGOGGCT GTGTACAG CGCAACAACG
3541 GCACCGGTCT GCTCCGAGCG CTGGAGATGA TTCTGGGTGG GCGTAGCGTA CCGGCTGATG
3601 AGGCGCTGAA GCTGGGTCTG ATCGATGCCA TTGCTACCGG CGATCAGGAC TCACTGTCCG
3661 TGGCATGCGC GTTAGCCCGT GCCCAATCG GCGCCGATGG TCAGTTGATC GAGTGGGCTG
3721 CGGTGACCCA GGCTTTCCGC CATCGCCACG AGCAGCTTGA CGAGTGGGCG AACCAGACC
3781 CCGGCTTTGC CGATGACGAA CTGCGCTCGA TTATGCCCCA TCCAGTATC GAGCGGATTA
3841 TCCGGCAGGC CCATACCGTT GGGCGCGATG CGGCAGTGCA TCGGCGACTG GATGCAATCC
3901 GCTATGGCAT TATCCACGGC TTGAGGCGG GTCTGGAGCA CGAGGCGAAG CTCTTGGCG
3961 AGGCAGTGGT TGACCCGAAC GGTGGCAAGC GTGGTATTGG CGAGTTCTC GACCGCCAGA
4021 GTGCGCCGTT GCCAACCCGC CGACCATGA TTACACCTGA ACAGGAGCAA CTCTGCGCG
4081 ATCAGAAAGA ACTGTTGCCG GTTGGTTTAC CCTTCTTCC CGGTGTTGAC CGGATTCCGA
4141 AGTGGCAGTA CGCGCAGGCG GTTATTGGTG ATCOGACAC CGGTGCGGCG GCTCACGGCG
4201 ATCCATCGT GCGTGAAGA CAGATTATTG TCGCGGTGGA ACGCCCCCG CCAATCAGG
4261 CGCTGATCTA TGTCTGGGCC TCGGAGGTGA ACTTCAACGA TATCTGGGCG ATTACCGGTA
4321 TTCCGGTGTG ACGGTTTGAT GAGCAGGACC GCGACTGGCA CGTTACCGGT TCAGGTGGCA
4381 TCGGCTGAT CGTTGCGCTG GGTGAAGAGG CGCGACGCGA AGGCGCGCTG AAGTGGGTG
4441 ATCTGGTGGC GATCTACTCC GGGCAGTCGG ATCTGCTCTC ACCCGTATG GCGCTGATC
4501 CGATGGCCGC CGATTTGCTC ATCCAGGGGA ACGACACGCC AGATGGATCG CATCAGCAAT
4561 TTATGCTGGC CCAGGCCCGG CAGTGTCTGC CCATCCCAAC CGATATGTCT ATCAGGCGAG
4621 CCGGCAGCTA CATCTCAAT CTGGTACGA TCTATGCGC CCTCTTTAG ACGTTGCCAA
4681 TCARGGCCGG ACGCAACATC TTTATCGAGG GTGCGGGGAC CGGTACCGGT CTGGACGCG
4741 CGGCTCGGC GGCOCGGAAT GGTCTGCGCG TAATTGGAAT GGTGAGTTG TCGTCAGGTG
4801 CGTCTACGCT GCTGGCTGCG GGTGCCACG GTGCGATTAA CCGTAAAGAC CCGGAGGTG
4861 CCGATTGTTT CACGCGGCTG CCCGAAGATC CATCAGCTG GGCAGCGCTG GAAGCGCGCG
4921 GTCAGCCGTT GCTGGCGATG TTCCGGGCGC AGAACGACGG GCGACTGGCC GATTATGTGG
4981 TCTCGCACGC GGGCGAGACG GCCTTCCCGC GCAGTTTCCA GCTTCTCGGC GAGCCACGCG
5041 ATGGTCACAT TCCGACGCTC ACATTCTAG GTGCCACCAG TGGCTACCAC TTCACCTTCC
5101 TGGGTAAAGC AGGCTCAGCT TCGCCGACCG AGATGCTGCG GCGGGCCAA CTCCGCGCG
5161 GTGAGGCGGT GTTATCTAC TACGGGGTTG GGAGCGATGA CCTGGTAGAT ACGGGGGTTC
5221 TGGAGGCTAT CGAGGCGGCG CGGCAATAGG GAGCGCGGAT CGTCTGCTG ACCGTACGCG
5281 ATGCGCAACG CGAGTTTGTG CTCTGTTGG GCTTGGGGC TGCCCTAAGT GGTGCTGCA
5341 GCCTGGCGGA ACTCAACCGG CGCTTCCGCG ATGAGTTTGA GTGGCCGCGC ACGATGCCGC
5401 CGTTGCCGAA CGCCCGCCAG GACCCGACGG GTCTGAAAGA GGCTGTCCG CGCTTCAACG
5461 ATCTGGTCTT CAAGCCGCTA GGAAGCGCGG TCGGTGTCTT CTGCGGAGT GCGACAATC
5521 CGCGTGGCTA CCCCAGCTG ATCATGAGC GGGCTGCCA CGATGCACTG GCGGTGAGCG
5581 CGATGCTGAT CAAGCCCTC ACCGGACGGA TTGTCTACTT CGAGGACATT GGTGGGCGGC
5641 GTTACTCCTT CTTCGCACCG CAAATCTGGG TCGGCCAGOG CCGCATCTAC ATGCCGACGG
5701 CACAGATCTT TGGTACGCAC CTCTCAAATG CGTATGAAAT TCTGCGTCTG AATGATGAGA
5761 TCAGCGCCGG TCTGCTGAGC ATTAOCGAGC CGGCAGTGGT GCGCTGGGAT GAACTACCGG
5821 AAGCACATCA GCGATGTGG GAAAATCGCC ACACGGCGGC CACTTATGTG GTGAATCATG
5881 CCTTACCAGC TCTCGGCTA AAGAACAGGG ACGAGCTGTA CGAGGCGTGG ACGGCGGGCG
5941 AGCGGTAGCG CGGATGGGTA TTGAACAGGT AACGGACGGA AGATCGAACC TTCCGTCCGT
6001 TATCTTTTGG CCGTGAAGC GTGCTGAGCC GATTATCGTT GCGGTGGTTG TCCGATGGG
6061 CAGACGCGCT CGAACAGAT GATACACCG ACGGCTATCG TCACCAACC GCGAAGACC
6121 AGGTAAGCCT CTGAAGGAC C (SEQ ID NO:38)

SUBSTITUTE SHEET (RULE 26)

39/105

Figure 28

```

1  MIDTAPLAPP RAPRSNPIRD RVDWEAQRAA ALADPGAFHG AIARTVIHWY DPOHHCWIRF
61  NESSQRWEGL DAATGAPVTV DYPADYQFWQ QAFDDSEAPF YRWFSGGLTN ACFNEVDREHV
121 MMGYGDEVAY YFEGDRWDNS LNNRGGGPVV QETITRRRL VEVVKAQVL RDLGLKKGDR
181 IALNMPNIMP QIYYTEAAKR LGILYTPVFG GFSDKTLSR IHNAGARVVI TSDGAYRNAQ
241 VVPYKEAYTD QALDKYIPVE TAQAIVAQTL ATLPLTESQR QTIITEVEAA LAGEITVERS
301 DVMRGVGSAL AKLRDLASV QAKVRTVLAQ ALVESPPRVE AVVVVRHTGQ EILWNEGRDR
361 WSHDLLDAAL AKILANARAA GFDVHSENDL LNLPPDQLIR ALYASIPCEP VDAEYPMFII
421 YTSGSTGKPK GVIHVHGGYV AGVVHTLRVS FDAEPGDTIY VIADPGWITG QSYMILTATMA
481 GRLTGVIAGS SPLFPSAGRY ASIIERYGVQ IFKAGVTFLK TVMSNPQNVV DVRLYDMHSL
541 RVATFCAEPV SPAVQQFGMQ IMTPQYINSY WATEHGGIVW THFYGNQDFP LRPDAHTYPL
601 PWVMGDVWVA ETDESGTTRY RVADFDERGE IVITAPYPYL TRTLWGDVPG FEAYLRGEIP
661 LRAWKGDAER FVKTYWRRGP NGENGYIQGD FAIKYPDGSF TLHGPRDDVI NVSGHRMGTE
721 EIEGAILRDR QITPDSFVGN CIVVGAPHRE KGLTPVAFIQ PAPGRHLTGA DRRRLDELVR
781 TEKGAVSVFE DYIEVSAPFE TRSGKYMRRF LRNMMLDEPL GDTTTLRNPE VLEEIAAKIA
841 EWKRQRMAE EQOIIERYRY FRIEYHPPTA SAGKLAVTVV TNPPVNALNE RALDELNTIV
901 DHLARRQDVA AIVFTGQGAR SFVAGADIRO LLEEHTVEE AMALPNNALH AFRKIERMKN
961 PCIAAINGVA LGGGLFAMA CHYRVADVYA EFGQPEINLR LLPGYGGTOR LPRLLYKRNK
1021 GTGLLRALEM ILGGRSVPAD EALKLGLIDA IATGDDQSL S LACALARA AI GADGQIESA
1081 AVTQAFRRHH EQLDEWRKPD PRFADDELRS IIAHPRIERI IRQAHTVGRD AAVHRAIDAI
1141 RYGIHGFEEA GLEHEAKLFA EAVVDPNGGK RGIREFLDRQ SAPLPTRRPL ITPEQEQLLR
1201 DQKELLFVGS PFFPGVDRIK KWQYQAVIR DPTGAAAHG DPIPAAEQII VFVERPRANQ
1261 ALIYVLASEV NFNDIWAITG IPVSRFDEHD RDWHVTGSGG IGLIVALGEE ARREGRLKVG
1321 DLVAIYSGQS DLSPIMGLD PMAADFVIQG NDTPDGSHQQ FMLAQAPQCL PIPTDMSIEA
1381 AGSYIINLGT IYRALFTTLQ IKAGRTIFIE GAATGTGLDA ARSAARNGLR VIGMVSSSSR
1441 ASTLLAAGAH GAINRKDFEV ADCFTRVPED PSANAWEAA GQPLLAMFRA QNDGRLADYV
1501 VSHAGETAFP RSFOLLGEPR DGHIPTLTFY GATSGYHFTF LGKPGSASPT EMLRRANLRA
1561 GEAVLIYYGV GSDDLVDVGG LEAIEAAROM GARIVVVTVS DAQREFVLSL GFGAALRGVV
1621 SLAELKRRFG DEFEPRTMP PLPNARQDPQ GLKEAVRRFN DLVFKPLGSA VGVFLRSADN
1681 PRGYPDIIIE RAAHDALAVS AMLIKPFTGR IVYFEDIGGR RYSEFFAPQIW VRQRIYMPY
1741 AQIFGTHLSN AYEILRLNDE ISAGLLTITE PAVVPWDELF EAHQAMWENR HTAATYVVNH
1801 ALPRGLKKNR DELYEAWTAG ER (SEQ ID NO:39)

```

SUBSTITUTE SHEET (RULE 26)

40/105

Figure 29

ATGAGTGAAGAGTCTCTGGTTCTCAGCACAATTGAAGGCCCCATCGCCATCCTCAACCTC
AATCGCCCCCAGGCCCTCAATGGGCTCAGTCCGGCCTTGATTGATGACCTCATTGCGCAT
TTAGAAGCCTGCGATGCCGATGACACAATCCGCGTGATCATTATCACCGCGCGCGGACGG
GCATTGCTGCCGGCGCTGATATCAAAGCGATGGCCAATGCCACGCCATTGATATGCTC
ACCAGTGGCATGATTGCGCGCTGGGCACGCATCGCCGCGGTGCGCAAACCGGTGATTGCT
GCCGTGAATGGGTATGCGCTCGGTGGTGGTTGTGAATTGGCAATGATGTGCGACATCATC
ATCGCCAGTGA AACGCGCAGTTCGGACAACGGGAAATCAATCTGGGCATCATTCCCGGT
GCTGGTGGCACC AACGGCTGACCGCGCCCTTGGCCCGTATCGCGCAATGGAATTGATC
CTGACCGGCGCGACCATCAGTGCTCAGGAAGCTCTCGCCACGGCCTGGTGTGCCGGGTC
TGCCCGCCTGAAAGCCTGCTCGATGAAGCCCGTCGGATCGCGCAAACCATTGCCACCAA
TCACCACTGGCTGTACAGTTGGCGAAAGAGGAGTCCGTATGGCCGCCGAAACCACTGTG
CGCGAGGGGTTGGCTATCGAGCTGCGTAACTTCTATCTGCTGTTTGCCAGTGCTGACCAA
AAAGAGGGGATGCAGGCATTATCGAGAAACGCGCTCCCAACTTCAGTGGTCGTTGA
(SEQ ID NO: 40)

41/105

Figure 30

MSEESLVLSTIEGPIAILTLNRPOALNALSPALIDDLIRHLEACDADDTIRVIIITGAGR
AFAAGADIKAMANATPIDMLTSGMIARWARIAAVRKPVIAAVNGYALGGGCELAMMCDII
IASENAQFGQPEINLGIIPGAGGTQRLTRALGPYRAMELILTGATISAOEALAHGLVCRV
CPPESLLDEARRIAQTIATKSPLAVQLAKEAVRMAAETTVREGLAIELRNFYLLFASADQ
KEGMQAFIEKRPNFSGR (SEQ ID NO: 41)

SUBSTITUTE SHEET (RULE 26)

42/105

Figure 31

GGCGTAATCCGACCGGCAGGTTAGGGTCTTCTACTGGGGTCAAGGCGCGTCTCCTTTTGG
TGGCGCGAGCAACCCGGCTTTTCTGGCTTCAATGTACCATAGAGCGGTTACTTCGTGCA
ACGGGCGTGGTACAATCGAGAGCAACCTTTTCGCAAAAGCTATCCAATCCTGCACACGTGC
ATCTGTTACAGGGTATTATTGTCTGGCAACGACAGTCTGTGCTTTATGTACAAGGAGAT
CAACGTATGAGTGAAGAGTCTCTGGTTCTCAGCACAAATTGAAGGCCCCATCGCCATCCTC
ACCCTCAATCGCCCCCAGGCCCTCAATGCGCTCAGTCCGGCCTTGATTGATGACCTCATT
CGCCATTTAGAAGCCTGCGATGCCGATGACACAATCCCGTGATCATTATCACCGGCGCC
GGACGGGCATTGCTGCCGGCGCTGATATCAAAGCGATGGCCAATGCCACGCCTATTGAT
ATGCTCACCAGTGGCATGATTGCGCGCTGGGCACGCATCGCCGCGGTGCGCAAACCGGTG
ATTGCTGCCGTGAATGGGTATGCGCTCGGTGGTGGTTGTGAATTGGCAATGATGTGCGAC
ATCATCATCGCCAGTGAAAACGCGCAGTTCGGACAACCGGAAATCAATCTGGGCATCATT
CCCGGTGCTGGTGGCACCCAACGGCTGACCCGCGCCCTTGGCCCGTATCGCGCAATGGAA
TTGATCCTGACCGGCGCGACCATCAGTGCTCAGGAAGCTCTCGCCACGGCCTGGTGTGC
CGGGTCTGCCCGCCTGAAAGCCTGCTCGATGAAGCCCGTCCGATCGCGCAAACCATTGCC
ACCAAATCACCCTGGCTGTACAGTTGGCGAAAGAGGCAGTCCGTATGGCCGCCGAAACC
ACTGTGCGCGAGGGGTTGGCTATCGAGCTGCGTAACTTCTATCTGCTGTTTGCCAGTGCT
GACCAAAAAGAGGGGATGCAGGCATTTATCGAGAAACGCGCTCCCAACTTCAGTGGTCTG
TGATCACGCGCAGAACATGGCAGCAGGGGCAATACCTGCACGTACTGCTCCTGCCGCCA
TACTACCAGATGATCGAGCAGTAAAGGGTAAATACTCTATCAATCTGGCCAGATAAGCGG
TTGGGTAACAACGCAATGCTCCAAAGGAGACGATCATGGACATACACGAGCGATTGCGAT
CTCTCGAACGCGAAAATGCT (SEQ ID NO:42)

43/105

Figure 32

SEQ ID NO:40	1 -----atgagtga-----agagt-----
SEQ ID NO:43	1 -----atgacgta-----cgaaa-----
SEQ ID NO:44	1 atggccgcccctgcgtgt-----cctgctgtcctgcgcccgcggcc
SEQ ID NO:45	1 atggcggcccctgcgtgtctgtctgtgccagagc-----
SEQ ID NO:40	14 -----ct-----ctg-----gttctc-agcacaattgaa
SEQ ID NO:43	14 -----cc-----atc-----ctggtcgagcgc-----gat
SEQ ID NO:44	41 cgctgaggccc-----ccg-----gttcgc-tgtccgcctgg
SEQ ID NO:45	33 -----ctgcaactcgctgtgtgtccccagttcgc-tgcccagaattc
SEQ ID NO:40	37 ggcccccatcgcc-----atcctcacc-----
SEQ ID NO:43	34 cagcgagttggc-----attatcacg-----
SEQ ID NO:44	73 cgtcccttcgcctcgggtgctaactttgagtacatcatcgcaaaaaag
SEQ ID NO:45	73 cggcgcttcgcctcgggtgctaactttcagttacatcatcacg-----
SEQ ID NO:40	58 -----c-----
SEQ ID NO:43	55 -----c-----
SEQ ID NO:44	123 agggaagaataaacaccgtggggtgatccaac-----
SEQ ID NO:45	115 -----gaaaagaaggaagaata
SEQ ID NO:40	59 -----tcaatcgccccaggccctcaatgcgctc
SEQ ID NO:43	56 -----tgaaccgtccccaggcactgaacgcgctc
SEQ ID NO:44	155 -----tgaaccgtccccaggccctcaatgcactt
SEQ ID NO:45	134 gcagcgtggggtgatccagttgaaccgtccccaaagcactcaatgcactt
SEQ ID NO:40	88 agtccggccttgattgatgacctcattc--gccatttagaagcctgcgat
SEQ ID NO:43	85 a--acagccagg--tgatgaacgaggtc--acca--gcgctgcaaccgaa
SEQ ID NO:44	184 tgcgatggcctgattgacgagctcaaccaggccctgaaga--tcttcgag
SEQ ID NO:45	184 tgcaatggactgattgaggagctcaacc--aagcactggagacctttgag
SEQ ID NO:40	136 ---gccgatgacaca---atccgcgtgatcattatcacccggcgccggacg
SEQ ID NO:43	127 ctggacgatgacccggacattggggcgatcatcatcacgggttcggccaa
SEQ ID NO:44	232 ---gaggaccggcc---gttggggcattgtcctcacccggcgggataa
SEQ ID NO:45	232 ---gaagatcccgct---gtgggcgcattgtgtcactggtggggagaa
SEQ ID NO:40	180 ggcatttgctgccggcgctgatatacaagcgatggccaa-----tgcc
SEQ ID NO:43	177 agcgtttgccgcccggagccgacatcaagaaatggccga-----cctg
SEQ ID NO:44	276 ggcctttgcagctggagctgatataaggaatgcagaacctgagtttcc
SEQ ID NO:45	276 ggcctttgcagccggagctgacatcaaggaatgcagaa-----ccgg
SEQ ID NO:40	223 acgcctattgatatgctcaccagtgccatgattgcgcgc---tggggcacg
SEQ ID NO:43	220 acgttcgcccagcgcttcaccgcccacttcttcgccacc---tggggcaa
SEQ ID NO:44	326 aggactgtt-----actccagcaagttcttgaagcac---tggggcca
SEQ ID NO:45	319 acatttcagga-ctgttactca--ggcaagttcctgagccactgggacca
SEQ ID NO:40	270 catcgccgcggtgcgcaaacgggtgattgctgccgtgaatgggtatgcgc
SEQ ID NO:43	267 gctggccgcgctgcgcaccccgacgatcgccgcggtggcgggatacgcgc
SEQ ID NO:44	366 cctcaccaggtcaagaagccagtcacgtcgtgtgcaatggctatccgt
SEQ ID NO:45	366 tatcaccggatcaagaaacgggtcatcgcggtgtcaatggctatgctc
SEQ ID NO:40	320 tcggtggtggtgtgaattggcaatgatgtgcgacatcatcatcgccagt
SEQ ID NO:43	317 tcggcggtggctgcgagctggcgatgatgtgcgacgtgctgatcgccgcc
SEQ ID NO:44	416 ttggcggggctgtgagcttgccatgatgtgtgatcatctatgcgggt
SEQ ID NO:45	416 ttggtgggggctgtgaacttgccatgatgtgcgatcatctatgctggt

SUBSTITUTE SHEET (RULE 26)

44/105

SEQ ID NO:40	370	gaaaacgcgcagttcggacaaccggaatcaatctgggcatcattcccgg
SEQ ID NO:43	367	gacaccgcgaagttcggacagcccagataaagctgggcgtgctgccagg
SEQ ID NO:44	466	gagaaggcccagtttgacacgcccggagatcttaataggaaccatcccagg
SEQ ID NO:45	466	gagaaagcccagtttggaagccagaaatcctcctggggaccatcccagg
SEQ ID NO:40	420	tgctgggtggcaccacaacggctgaccgcgccccttgcccgtatcgcgcaa
SEQ ID NO:43	417	catgggcggctcccagcggctgaccggggtatcggaaggctaaggcga
SEQ ID NO:44	516	tgacggcggcaccacagagactcaccggtgctgttggaagtgcgtggagc
SEQ ID NO:45	516	tgacgggggcactcagagactcaccgagcagtcggcaaatcactagcaa
SEQ ID NO:40	470	tggattgatcctgaccggcgccagcatcagtgctcaggaagctctcgcc
SEQ ID NO:43	467	tggacctcatcctgaccggcgccacatggacgcgcggcaggc-cgagcg
SEQ ID NO:44	566	tggagatggctcctcaccggtgacgcgatctcagcccaggacgc-caagca
SEQ ID NO:45	566	tggagatggctcctcactggtgaccgaatttcagcacaggtatgc-caagca
SEQ ID NO:40	520	ca-c-ggcctggtgtgcccgggtctgcccgcctgaaagcctgctcgatgaa
SEQ ID NO:43	516	cagc-ggtctggtttcacgggtggtgcccggcgacgacttgctgaccgaa
SEQ ID NO:44	615	ag-caggtcttgtagcaagatttgctcctgttgagacactggtggaagaa
SEQ ID NO:45	615	ag-caggtcttgtagcaagattttcccggtgaaacactggttgaaagag
SEQ ID NO:40	568	gcccgtcggatcgcgcaaacattgccaccaaatcaccactggctgtaca
SEQ ID NO:43	565	gccagggccactgccacgaccatttcgcagatgtcgccctcggcgggccc
SEQ ID NO:44	664	gccatccagtgtgcagaaaaattgccagcaattctaaaattgtagtgc
SEQ ID NO:45	664	gccatccaatgtgcagaaaagatcgccaacaattccaagatcatagtgc
SEQ ID NO:40	618	gttggcgaaagaggcagtcggtatggccgcccgaaccactgtgcgcgagg
SEQ ID NO:43	615	gatggccaaggaggccgtcaaccgggctttcgaaatccagtttgctccgagg
SEQ ID NO:44	714	gatggccaagaatcagtgaaatgcagcttttgaatgacattaacagaag
SEQ ID NO:45	714	catggcgaaagaatctgtgaatgcagcctttgaaatgacgttaacagaag
SEQ ID NO:40	668	ggttggctatcgagctgcgtaacttctatctgctgtttgccagtgtgcac
SEQ ID NO:43	665	ggctgctctacgaacgcccggctttccattcggttttcgcgaccgaagac
SEQ ID NO:44	764	gaagtaagttggagaagaaactcttttattcaacctttgccactgatgcac
SEQ ID NO:45	764	gaaataagctggagaagaagctcttctattccacctttgccactgatgcac
SEQ ID NO:40	718	caaaaagaggggatgcaggcatttatcgagaaacgcgctcccaacttcag
SEQ ID NO:43	715	caatccgaagggtatggcagcgttcacgagaaacgcgctccccagttcac
SEQ ID NO:44	814	cggaaagaagggtatgaccgcgtttgtggaaaagagaaaggccaacttcaa
SEQ ID NO:45	814	cggagagaagggtatgtctgcctttgtggagaaaagggaaggccaacttcaa
SEQ ID NO:40	768	tggtcgttga
SEQ ID NO:43	765	ccaccgatga
SEQ ID NO:44	864	agaccagtga
SEQ ID NO:45	864	agaccactga

SUBSTITUTE SHEET (RULE 26)

45/105

Figure 33

SEQ ID NO:41	1 -mseeelv-----lstiegp-----
SEQ ID NO:46	1 -mtyetil-----ver-dqr-----
SEQ ID NO:47	1 -maalrvl-----lscargplppvrpcawrpfasganfeyiiaekrg
SEQ ID NO:48	1 maalrallpracnslspvrceffrfasganfgyiitekkgkns--
SEQ ID NO:41	15 ----iailtlnrpqalnalspaliddlirhleacdaddtirviitgagr
SEQ ID NO:46	14 ----vgiitlnrpqalnalsqvmnevtsaateldddpdigaiiitgsak
SEQ ID NO:47	43 knntvgliqlnrpkalnalcldglidelnqalkifeedpavgaivltggdk
SEQ ID NO:48	47 ----vgliqlnrpkalnalcnglieelnqaletfeedpavgaivltggek
SEQ ID NO:41	61 afaagadikamanatpidmltsgmiarwariaavrkpviaavngyalggg
SEQ ID NO:46	60 afaagadikemadltfadaftadffatwgklaavrtptiaavagyalggg
SEQ ID NO:47	93 afaagadikemqnlsfqdcysgkflkhwdhltqvkpviaavngyafggg
SEQ ID NO:48	93 afaagadikemqnrftqdcysgkflshwdhitrikpviaavngyalggg
SEQ ID NO:41	111 celammcdiiiasenaqfgqpeinlgiipgaggtqrltralgyprameli
SEQ ID NO:46	110 celammcdvliiaadtakfgqpeiklgvlpmggsgqrltraigkakamdli
SEQ ID NO:47	143 celammcdiiyagekaqfaqpeillgtipgaggtqrltravgkslamemv
SEQ ID NO:48	143 celammcdiiyagekaqfgqpeillgtipgaggtqrltravgkslamemv
SEQ ID NO:41	161 ltgatisaqealahglvcrvcppeslldearriaqtiatksplavqlake
SEQ ID NO:46	160 ltgrtmdaaeaersglvsrvvpaddlilearatattisqmsasaarmake
SEQ ID NO:47	193 ltgdrisaqdaqaglvsklcpvetlveeaiqcaekiasnsvivamake
SEQ ID NO:48	193 ltgdrisaqdaqaglvsklfvvetlveeaiqcaekiansskiivamake
SEQ ID NO:41	211 avrmaaeettvreglaielrnfyllfasadqkegmqafiekrapnfsgr
SEQ ID NO:46	210 avnrafesslseglllyerrlfsaafatedqsegmaafiekrapqfthr
SEQ ID NO:47	243 svnaafemtlttegskleklfystfatddrkegmtafvekrkanfkdk
SEQ ID NO:48	243 svnaafemtlttegnkleklfystfatddrregmsafvekrkanfkdh

SUBSTITUTE SHEET (RULE 26)

1. Importance

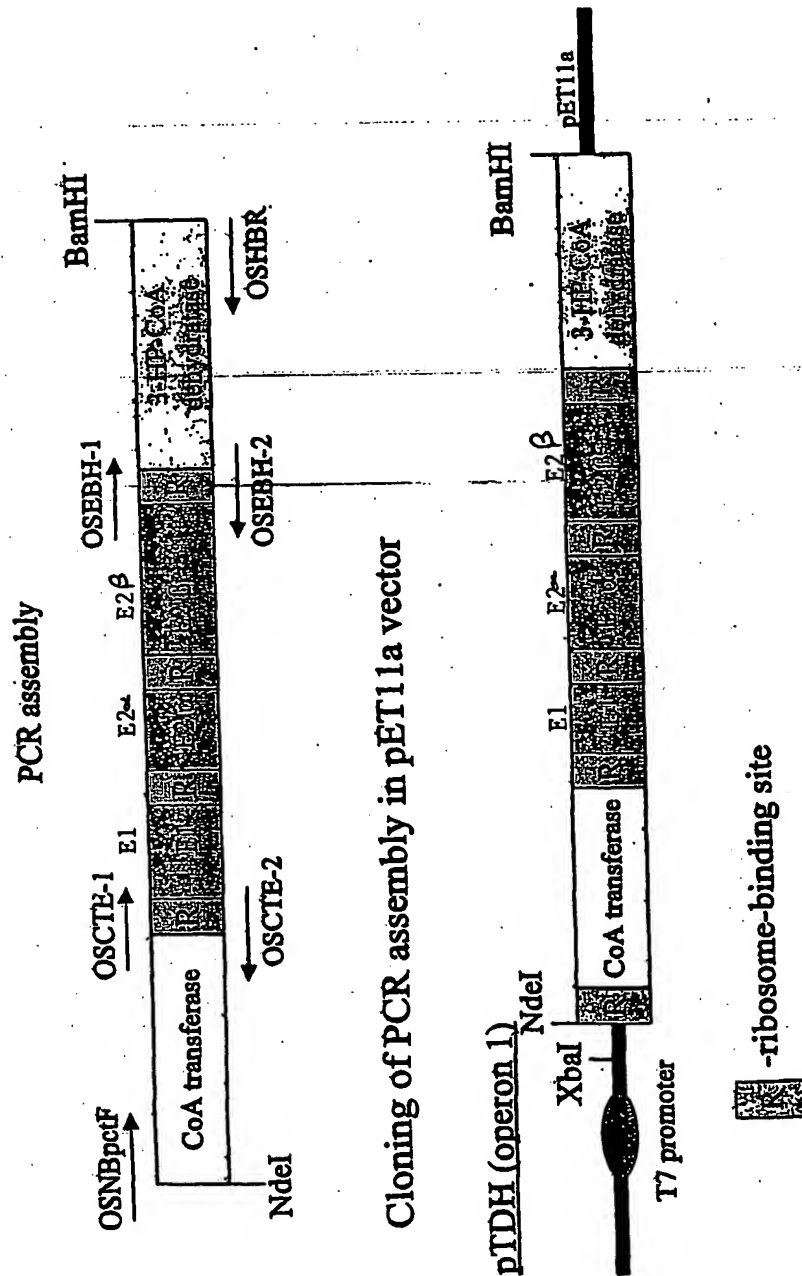
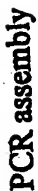


Figure 35A

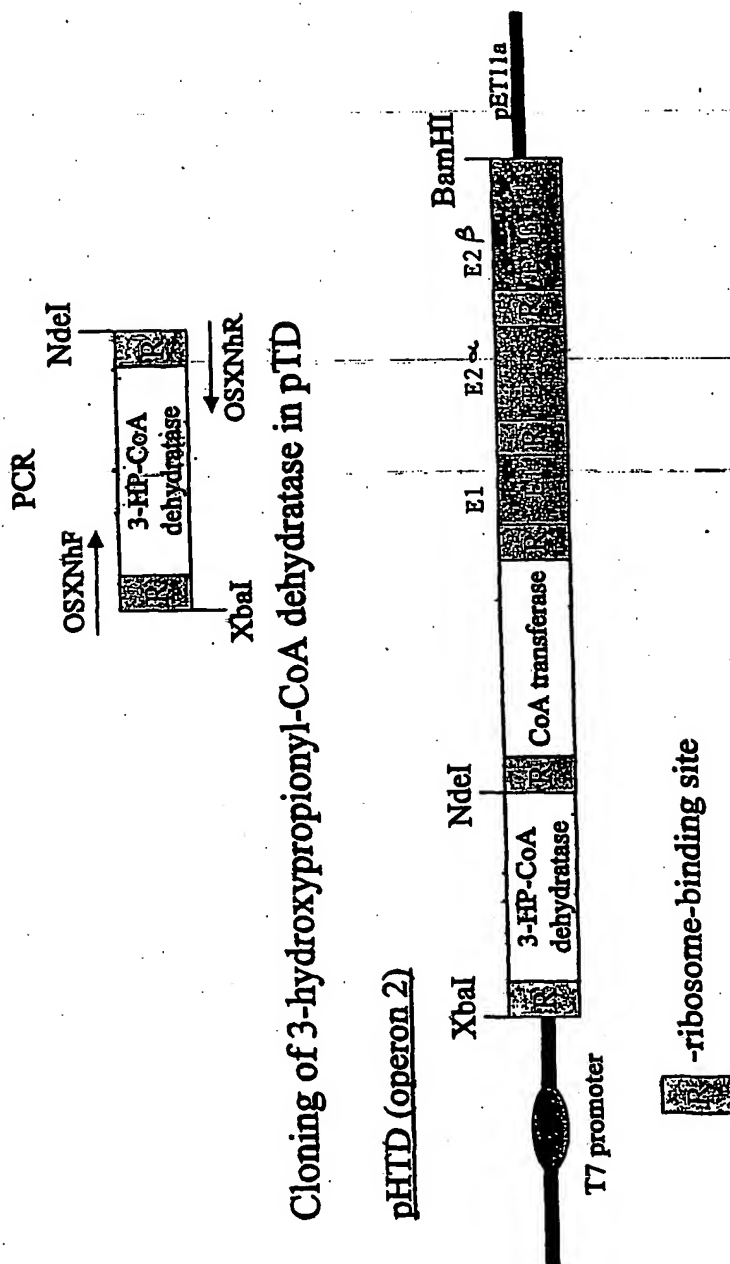


Cloning of PCR assembly in pET11a vector



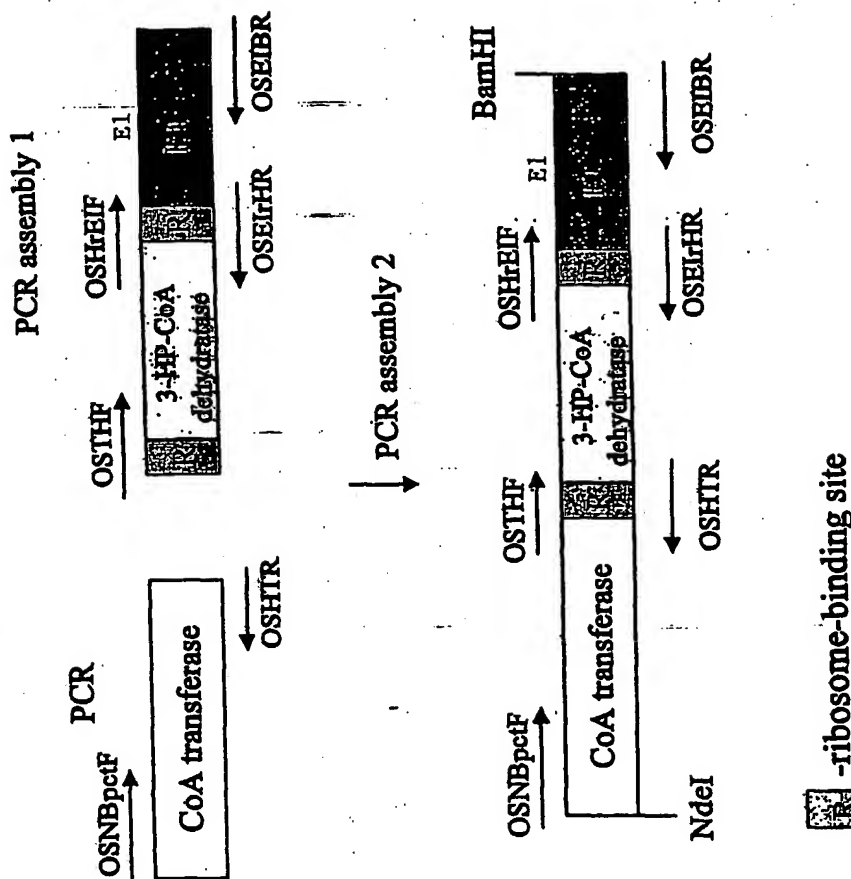
48/105

Figure 30B



49/105

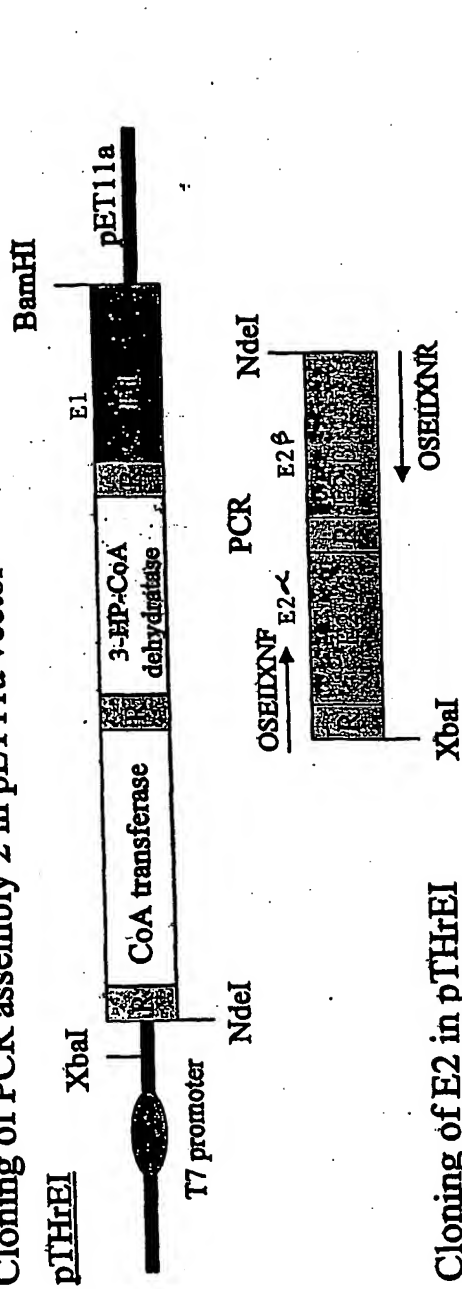
Figure 36A



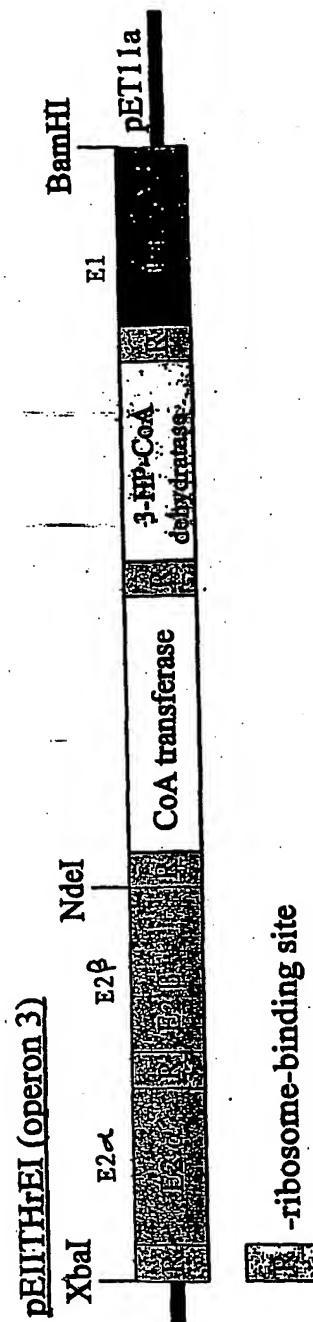
50/105

Figure 36B

Cloning of PCR assembly 2 in pET11a vector

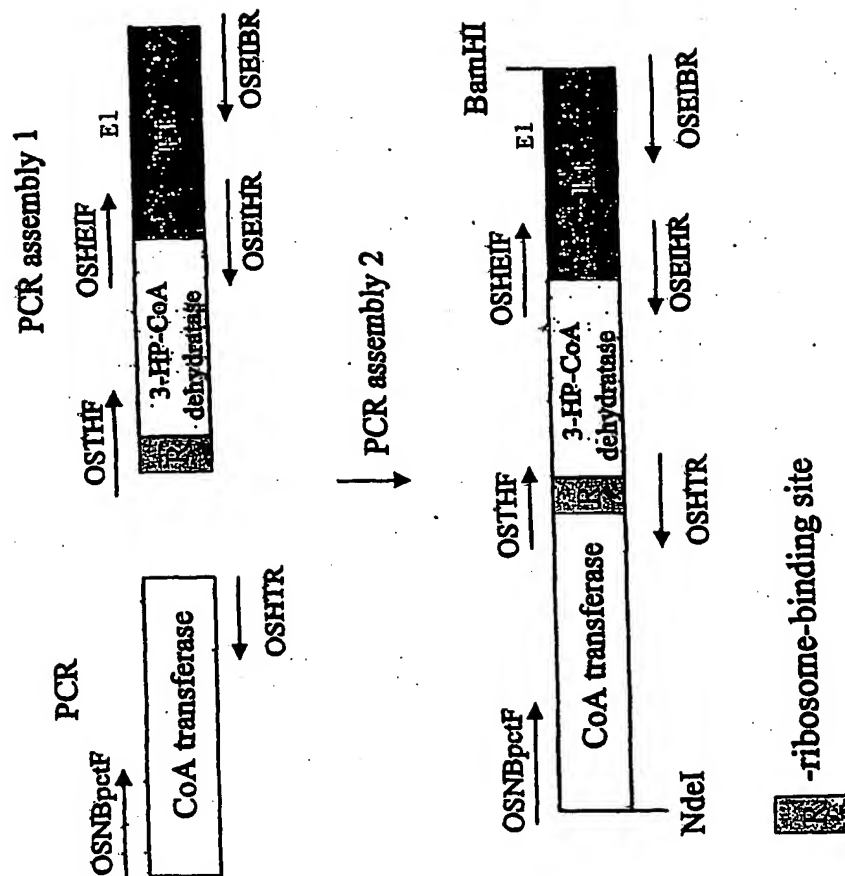


Cloning of E2 in pTHrEI



51/105

Figure 3/A

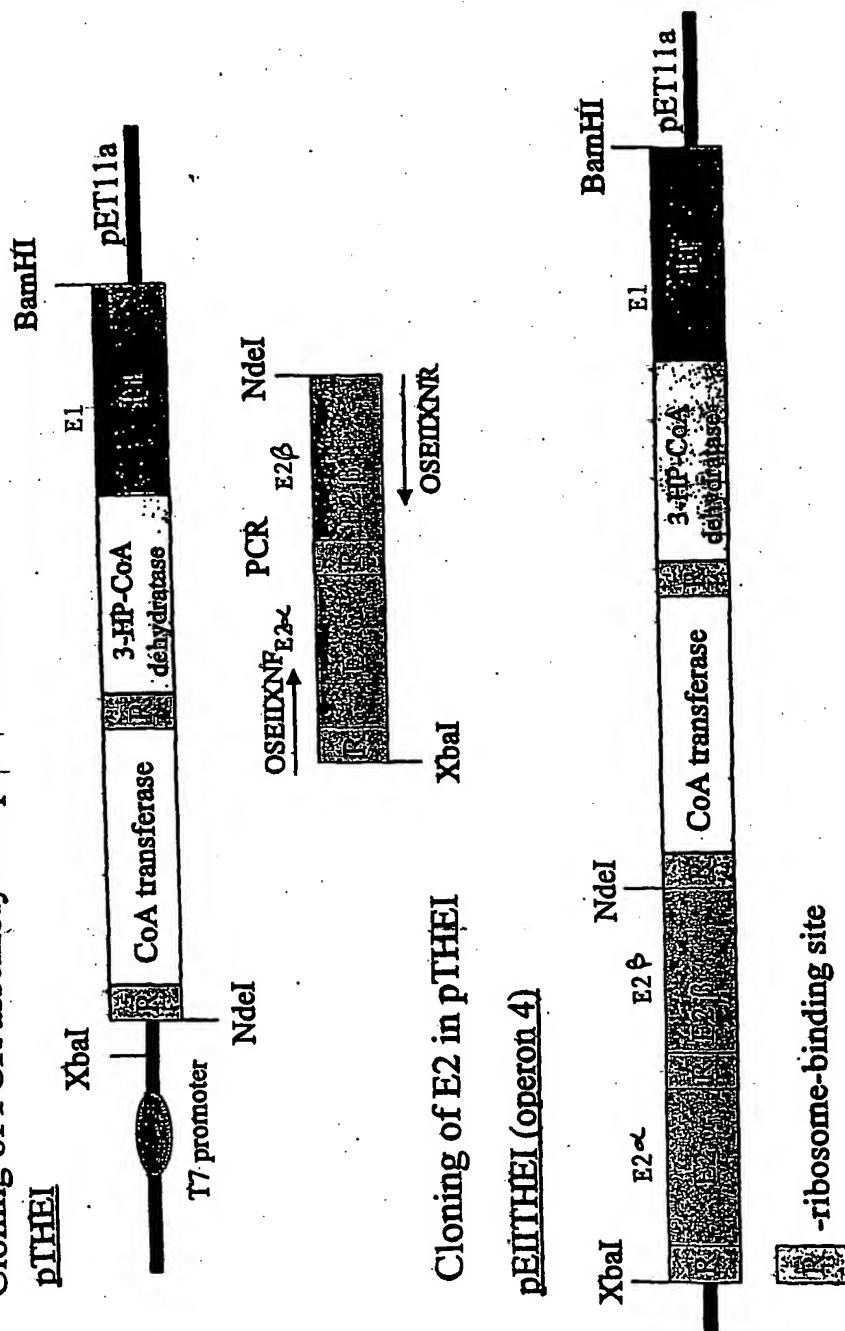


SUBSTITUTE SHEET (RULE 26)

52/105

Figure 3 / B

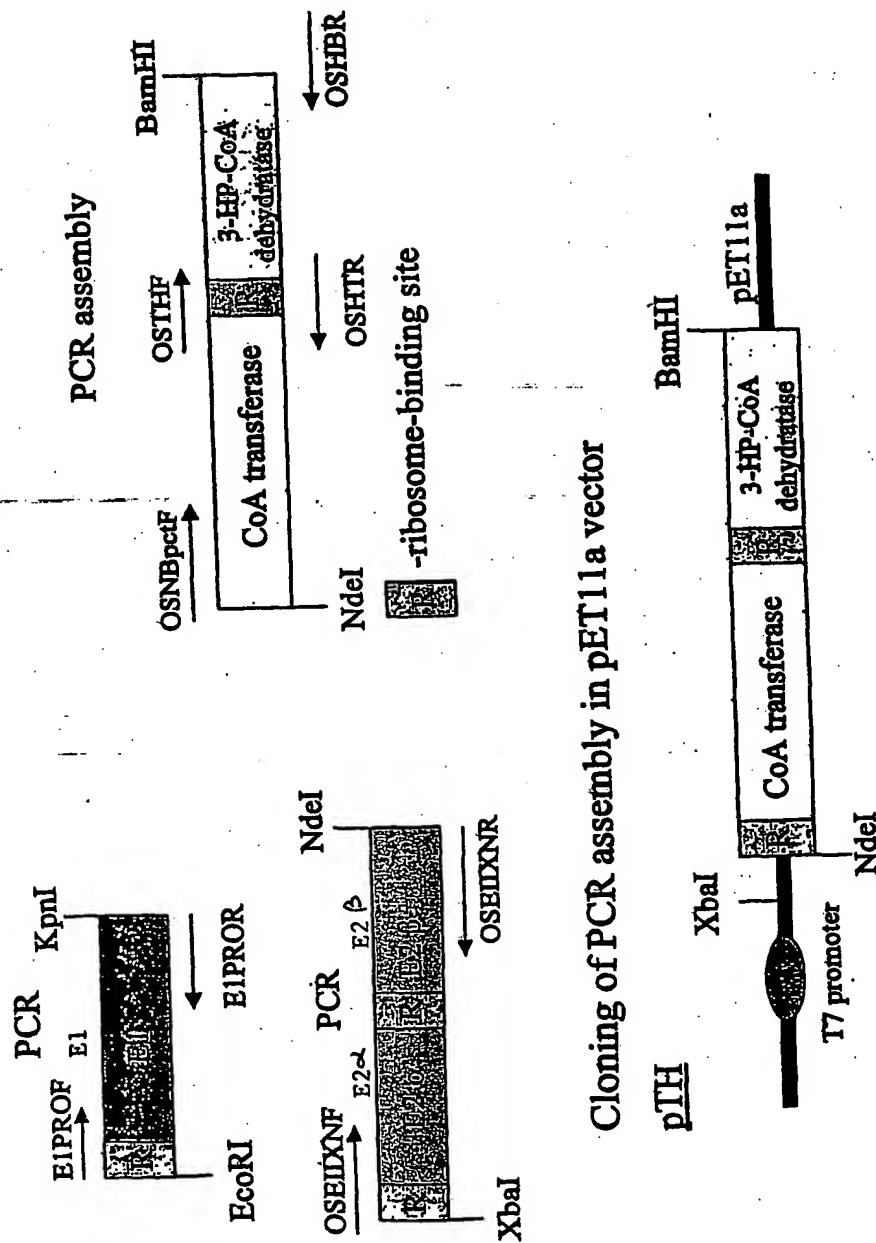
Cloning of PCR assembly 2 in pET11a vector



SUBSTITUTE SHEET (RULE 26)

53/105

Figure 38A

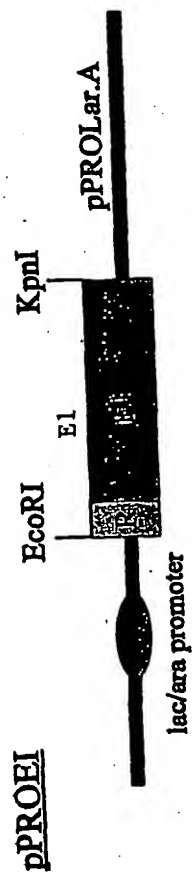


SUBSTITUTE SHEET (RULE 26)

54/105

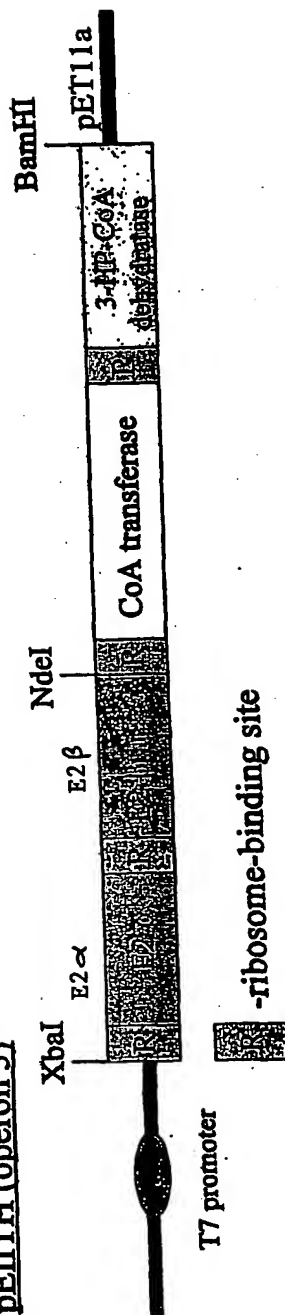
Figure 30D

Cloning of E1 gene separately in pPROLar.A vector



Cloning of E2 in pTH

pE11TH (operon 5)



55/105

Figure 39

ATGATCGACACTGCGCCCCCTTGCCCCACCACGGGGCGCCCGCTCTAATCCGATTTCGGGAT
CGAGTTGATTGGGAAGCTCAGCGCGCTGCTGCGCTGGCAGATCCCGGTGCCTTTTCATGGC
GCGATTGCCCCGACAGTTATCCACTGGTACGACCCACAACACCATTTGCTGGATTTCGCTTC
AACGAGTCTAGTCAGCGTTGGGAAGGGCTGGATGCCGCTACCGGTGCCCTGTAAACGGTA
GACTATCCCGCCGATTATCAGCCCTGGCAACAGGCGTTTGTATGATAGTGAAGEGCGCTTT
TACCGCTGGTTTAGTGGTGGGTTGACAAATGCCTGCTTTAATGAAGTAGACCGGCATGTC
ATGATGGGCTATGGCGACGAGGTGGCCTACTACTTTGAAGGTGACCGCTGGGATAACTCG
CTCAACAATGGTCGTGGTGGTCCGGTTGTCCAGGAGACAATCACGCGGCGGCGCCTGTTG
GTGGAGGTGGTGAAGGCTGCGCAGGTGTTGCGTGATCTGGCCCTGAAGAAGGGTGATCGG
ATTGCTCTGAATATGCCGAATATTATGCCGAGATTTATTATACGGAAGCGGCAAAACGA
CTGGGTATTCTGTACACGCCGGTCTTCGGTGGCTTCTCGGACAAGACTCTTTCCGACCGT
ATTCACAATGCCGGTGCACGAGTGGTGATTACCTCTGATGGTGGTACCGCAACGCGCAG
GTGGTGCCCTACAAAGAAGCGTATACCGATCAGGGGCTCGATAAGTATATTCGGGTTGAG
ACGGCGCAGGCGATTGTTGCGCAGACCCTGGCCACCTTGCCCTGACTGAGTCGEAGCGC
CAGACGATCATCACCAGAGTGGAGGCGCGACTGGCCGGTGAGATTACGGTTGAGCGCTCG
GACGTGATGCGTGGGGTGGTTCTGCCCTCGCAAAGCTCCGCGATCTTGATGCAAGCGTG
CAGGCAAAGGTGCGTACAGTACTGGCGCAGGCGCTGGTGCAGTCGCGCGCGGGTTGAA
GCTGTGGTGGTTGTGCGTCATACCGGTGAGGAGATTTTGTGGAACGAGGGGCGAGATCGC
TGGAGTCACGACTTGCTGGATGCTGCGCTGGCGAAGATTCTGGCCAATGCGCGTGCTGCC
GGCTTTGATGTGCACAGTGAGAATGATCTGCTCAATCTCCCGATGACCAGCTTATCCGT
GCGCTCTACGCCAGTATTCCCTGTGAACCGGTTGATGCTGAATATCCGATGTTTATCATT
TACACATCGGGTAGCACCGGTAAGCCCAAGGGTGTGATCCACGTTACGGCGGTTATGTC
GCCGGTGTGGTGACACCTTGCGGGTCAGTTTTGACGCCGAGCCGGGTGATACGATATAT
GTGATCGCGGATCCGGGCTGGATCACCAGGTGAGAGCTATATGCTCACAGCCACAATGGCC
GGTCGGCTGACCGGGGTGATTGCCGAGGGATCACCCTCTTCCCTCAGCCGGGCGTTAT
GCCAGCATCATCGAGCGCTATGGGGTGAGATCTTTAAGCGGGTGTGACCTTCTCTCAAG
ACAGTGATGTCCAATCCGCAGAATGTTGAAGATGTGCGACTCTATGATATGCACTCGCTG
CGGGTTGCAACCTTCTGCGCCGAGCCGGTCAGTCCGGCGGTGACGAGTTTGGTATGCAG
ATCATGACCCCGCAGTATATCAATTTCGTAAGTGGGCGACCGAGCAGCGTGAATTGTCTGG
ACGCAATTTCTACGGTAATCAGGACTTCCCGCTTCGTCCCGATGCCCATACCTATCCCTTG
CCCTGGGTGATGGGTGATGCTGCGGTGGCCGAACTGATGAGAGCGGGACGACGCGCTAT
CGGGTCGCTGATTTGATGAGAAGGGCGAGATTGTGATTACCGCCCGTATCCCTACCTG
ACCCGCACACTCTGGGGTGATGTGCCCGGTTTCGAGGCGTACCTGCGCGGTGAGATTCCG
CTGCGGGCCTGGAAGGGTGATGCCGAGCGTTTCGTCAAGACCTACTGGCGAGCTGGGCCA
AACGGTGAATGGGGCTATATCCAGGGTGATTTGCCATCAAGTACCCCGATGGTAGCTTC
ACGCTCCACGGACGCCCTGACGATGTGATCAATGTGTCGGGCCACCGTATGGGCACCGAG
GAGATTGAGGGTGCCATTTTGGTGACCGCCAGATCACGCCGACTCGCCCGTGGTAAT
TGATTGTGGTGGTGCGCGCACCGTGAGAAGGGTCTGACCCCGGTTGCCCTTCAATCAA
CCTGCGCTGGCCGTCTCTGACCGGCGCCGACCGGCGCGCTCTCGATGAGCTGGTGCGT
ACCGAGAAGGGGGCGGTGAGTGTCCAGAGGATTACATCGAGGTGAGTCCCTTCCCGAA
ACCCGCAGCGGGAAGTATATGCCGGCGCTTTTGGCGCAATATGATGCTCGATGAACCACTG
GGTGATACGACGACGTTGCCGAATCTTGAAGTGCTCGAAGAGATTGCAGCCAAGATCGCT
GAGTGGAAACGCCGTGAGCGTATGGCCGAAGAGCAGCAGATCATCGAACGCTATCGCTAC
TTCCGGATCGAGTATCACCCACCAACGGCCAGTGCGGGTAACTCGCGGTAGTGACGGTG
ACAAATCCCGCGGTGAACGCACTGAATGAGCGTGCGCTCGATGAGTTGAACACAATTGTT
GACCACCTGGCCCGTCTGAGGATGTTGCCGCAATGTCTTACCGGACAGGGCGCCAGG
AGTTTTGTGCGCGGCGCTGATATTGCCAGTTGCTCGAAGAGATTACATCGGTTGAAGAG
GCAATGGCCCTGCCGAATAACGCCATCTTGCTTCCGCAAGATTGAGCGTATGAATAAG

SUBSTITUTE SHEET (RULE 26)

56/105

CCGTGTATCGCGGCGATCAACGGTGTGGCGCTCGGTGGTGGTCTGGAATTCGCCATGGCC
TGCCATTACCGGGTTGCCGATGTCTATGCCGAATTCGGTCAGCCAGAGATTAATCTGCGC
TTGCTACCTGGTTATGGTGGCACGCAGCGCTTGCCGCGCCTGTTGTACAAGCGCAACAAC
GGCACC GGCTGCTCCGAGCGCTGGAGATGATTCTGGGTGGGCGTAGCGTACCGGCTGAT
GAGGCGCTGAAGCTGGGTCTGATCGATGCCATTGCTACCGGCGATCAGGACTCACTGTCTG
CTGGCATGCGCGTTAGCCCGTGCCGAATCGGCGCCGATGGTCAGTTGATCGAGTCGGCT
GCGGTGACCCAGGCTTTCCGCCATCGCCACGAGCAGCTTGACGAGTGGCGCAAACCAGAC
CCGCGCTTTGCCGATGACGAACCTGCGCTCGATTATCGCCCATCCACGTATCGAGCGGATT
ATCCGGCAGGCCCCATACCGTTGGGCGCGATGCGGCAGTGCCATCGGGCACTGGATGCAATC
CGCTATGGCATTATCCACGGCTTCGAGGCGGCTCGGAGCAGGCGCAAGCTCTTTGCC
GAGGCAGTGGTTGACCCGAACGGTGGCAAGCGTGGTATTTCGCGAGTTCTCGACCGCCAG
AGTGCGCCGTTGCCAACCCGCGGACCATTGATTACACCTGAACAGGAGCAACTCTTGCGC
GATCAGAAAGAACTGTTGCCGGTTGGTTCACCCTTCTTCCCCGGTGTGACCGGATTCCG
AAGTGGCAGTACGCGCAGGCGGTTATTTCGTGATCCGGACACCGGTGCGGCGGCTCACGGC
GATCCCATCGTGGCTGAAAAGCAGATTATTGTGCCGGTGGAACGCCCCGCGCCAATCAG
GCGCTGATCTATGTTCTGGCCTCGGAGGTGAACCTCAACGATATCTGGGCGATTACCGGT
ATTCGGGTGTCACGGTTTGATGAGCACGACCGCGACTGGCACGTTACCGGTTACGGTGGC
ATCGGCCTGATCGTTGCGCTGGGTGAAGAGGCGCGACGCGAAGGCCGGCTGAAGGTGGGT
GATCTGGTGGCGATCTACTCCGGCAGTCGGATCTGCTCTACCGCTGATGGGCCTTGAT
CCGATGGCCGCGGATTTCGTATCCAGGGGAACGACACGCCAGATGGATCGCATCAGCAA
TTTATGCTGGCCCCAGCCCCGAGTGTCTGCCCATCCCAACCGATATGCTCTATCGAGGCA
GCCGGCAGCTACATCCTCAATCTCGGTACGATCTATCGCGCCCTCTTTACGACGTTGCAA
ATCAAGGCCGGACGCACCATCTTTATCGAGGGTGCGGCGACCGGTACCGGTCTGGACGCA
GCGCGCTCGGCGGCCCGGAATGGTCTGCGCGTAATTGGAATGGTCAGTTCGTGCTCACGT
GCGTCTACGCTGCTGGCTGCGGGTGCCCCAGGTGCGATTAACCGTAAAGACCCGGAGGTT
GCCGATTGTTTCACGCGCGTGCCCCGAAGATCCATCAGCCTGGGCAGCCTGGGAAGCCGCC
GGTCAGCCGTTGCTGGCGATGTTCCGGGCGCAGAACGACGGGCGACTGGCCGATTATGTG
GTCTCGCACGCGGGCGAGACGGCCTTCCCGCGCAGTTTCAGCTTCTCGGCGAGCCACGC
GATGGTCACATTCCGACGCTCACATTCTACGGTGCCACCAGTGGCTACCACTTCACCTTC
CTGGGTAAAGCCAGGGTCAGCTTCGCGGACCGAGATGCTGCGGCGGGCCAATCTCCGCGCC
GGTGAGGCGGTGTTGATCTACTACGGGTTGGGAGCGATGACCTGGTAGATACCGGCGGT
CTGGAGGCTATCGAGGCGCGCGGCAATGGGAGCGCGGATCGTCGTGCTTACCGTCAGC
GATGCGCAACGCGAGTTTGCTCTCTCGTTGGGCTTCGGGGCTGCCCTACGTGGTGTCTGTC
AGCCTGGCGGAACCTCAAACGGCGCTTCGGCGATGAGTTTGAGTGGCCGCGCACGATGCCG
CCGTTGCCGAACGCCCCGCCAGGACCCGCAGGGTCTGAAAGAGGCTGTCCGCGCTTCAAC
GATCTGGTCTTCAAGCCGCTAGGAAGCGCGGTGCGTGTCTTCTTGGGAGTGCCGACAAAT
CCGCGTGGCTACCCGATCTGATCATCGAGCGGGCTGCCACGATGCACTGGCGGTGAGC
GCGATGCTGATCAAGCCCTTACCGGACGGATTGTCTACTTCGAGGACATTGGTGGGCGG
CGTTACTCCTTCTTCGCACCGCAAATCTGGGTGCGCCAGCGCCGATCTACATGCCGACG
GCACAGATCTTTGGTACGCACCTCTCAAATGCGTATGAAATCTGCGTCTGAATGATGAG
ATCAGCGCCGGTCTGCTGACGATTACCGAGCCGGCAGTGGTGCCGTGGGATGAACTACCC
GAAGCACATCAGGCGATGTGGGAAAATCGCCACACGGCGGCCACTTATGTGGTGAATCAT
GCCTTACCACGTCTCGGCCATAAGAACAGGGACGAGCTGTACGAGGCGTGGACGGCGCGG
GAGCGGTAG (SEQ ID NO:129)

SUBSTITUTE SHEET (RULE 26)

57/105

Figure 40

SEQ ID NO:39	1	-----midtaplappraprsnpirdrvdwe
SEQ ID NO:130	1	mglpeervrsgsgsrgqeeagaggrarswsp--ppevrsahvpslqryr
SEQ ID NO:131	1	-----mslelkekeselpfdeqiind
		PL PP RS P
SEQ ID NO:39	26	aqraaaladpgafghgaiartvihwydpqhhcwifnessqrwegldaatg
SEQ ID NO:130	49	elhrzsveeprefwgdiake-fywktpcpgpflyrn-----
SEQ ID NO:131	22	kwrs-----kytpidayfkfhrqtvenlesf--wesv-----
		R P F G I A T I W Y P H R NES WE
SEQ ID NO:39	76	apvtvdypadyqpwwqafddseap-fyrwfsggltnacfnvdrhvm-mg
SEQ ID NO:130	84	-----fdvtkgkifiewmkgattnicynvldrnvhck
SEQ ID NO:131	52	-akelew---fkpwdkvldasnpp-fykwfvggrrlnslayldrvhvk-tw
		FW FD S P FY WF GG TN C N VDRHV
SEQ ID NO:39	124	ygdevayyfeqdrwdslnnrgggpvvqetitrllvevvkaaqylr-d
SEQ ID NO:130	117	lgdkvafywegne-----pgettqityhqllvqvcqfsanvlr-k
SEQ ID NO:131	96	rknklaiewegepvdn-----gyptdrkltyydlxevnrwaymlkqn
		GD VA Y EG D G P IT LLVEV A VLR
SEQ ID NO:39	173	lgkkkgdrialnmpnimpqiyyte-aakrlgilytpvfqggsdktlsdri
SEQ ID NO:130	155	qgiqkgdrvaitympmipelvaml-acarigalhsivfagfsseslceri
SEQ ID NO:131	141	fgvkkkgdkitlylp-mvpelpitmlaawrigaitsvvfagfsadalaeri
		G KKGDRIAL MP I P T AA R G L VF GFS L RI
SEQ ID NO:39	222	hnagarvvitsdgayrnaqvvpvpykeaytdqal----dkyipvetaqaiva
SEQ ID NO:130	204	ldsscallittdafyrgeklvnlkel-adealqkqekgfpvrc--civv
SEQ ID NO:131	190	ndsqsrivitadgfwrrgrvvrkew-----
		R VIT DG YR VV KE D AL K PV IV
SEQ ID NO:39	268	qtlatlplesqrqtiiteveaalageitversdvmrgvgsalaklrdd
SEQ ID NO:130	251	khlgrael-----gmgdsts
SEQ ID NO:131	216	-----vdaal-----
		L L V AAL G G
SEQ ID NO:39	318	asvqakvrtvlaqalvespprveavvvvrhtg-qeilwnegrdrwshdl1
SEQ ID NO:130	266	-----qsppikrscpdv-----qiswnqgidlwwhelm
SEQ ID NO:131	221	-----ekatqvesvivlprlgkdvpmtegrdywnklm
		ESPP VE V VV G I WNEGRD W H L
SEQ ID NO:39	367	daalakilanaraagfdvhsendl1nlpddqliralyasipcep--vdae
SEQ ID NO:130	294	gea-----gde-----cepewcdae
SEQ ID NO:131	255	q-----gipn-----ayiepep--vese
		A P D A I CEP VDAE
SEQ ID NO:39	415	ypmfiiytsgstgkpgkpvihvhggyvagvvhtrvsfdaepgdtiyviad
SEQ ID NO:130	309	dplfilytsgstgkpgkvvhtvggmylvattfkyyvdfhaedvfwtad
SEQ ID NO:131	272	hpsfilytsgstgkpgkpvihvdtggwvhyatmkwvfdirdddifwtad
		P FI YTSSTGKPKGV H GGY V T FD D AD
SEQ ID NO:39	465	pgwitgqsymltatmagrltgviaegsplfpsagryasierygvqifka
SEQ ID NO:130	359	igwitghsyvtygplangatsvlfegiptypdvnrslwsivdkykvtkfyt
SEQ ID NO:131	322	igwvtghsyvvlqpllmgateviyegapdyppqdrwswsierygvttifyt
		GWITG SY A T VI EG P P R SIERYGV IF

SUBSTITUTE SHEET (RULE 26)

58/105

SEQ ID NO:39	515	gvtfllktvmsnpqnvdrlydmhslrvatfcaepvspavqqfgmqimtp
SEQ ID NO:130	409	aptairllmkfgd--epvtkhsraslqvlgtvgepinpeawlwyhrvvga
SEQ ID NO:131	372	sptairmfmyrge--ewprkhdltlrlihsvglpinpeawrwayrvlgn
		T M E VR D SLRV EP P
SEQ ID NO:39	565	q---yi---nsywaterhggivwthfygnqdfplrpdahtypplpwmqgdwv
SEQ ID NO:130	457	qrcpiv---dtfwqtetggghmltpipgat--pmkpgsatfp----ffgva
SEQ ID NO:131	420	e---kvafgstwmmtetggivishapglylvpmkpgtngpplpgfevdv-
		Q W TE GGIV TH G P P T PLP DV
SEQ ID NO:39	609	vaetdesgttryrvadfddekgeivtapypyltrtlwgdvpgfeaylrge
SEQ ID NO:130	498	pailnesg---eelegeaegylvfkqpwpgimrtvy-----
SEQ ID NO:131	466	---vdengnp---appgvkgylvikkpwpngmlhgiw-----
		A DESG A KG VI P P RT W
SEQ ID NO:39	659	iplrawkgdaerfvktywrrqpngewgylqgdfaikypdgsftlhgrpdd
SEQ ID NO:130	531	-----gnherfettyfkkfpg---yyvtgdgcqrdqdywitgridd
SEQ ID NO:131	496	-----gdperyktywxfpg---mfyagdyaikdkdgyiwlgrade
		GD ERF KTYW R P Y GD AIK DG GR DD
SEQ ID NO:39	709	vinvsghrmgteeelegailrdrqitpdsfvncivvgaphrekgltpvaf
SEQ ID NO:130	571	mlnvsghllstaevesalve-----heavaeaaavvgphpvkgeclycf
SEQ ID NO:131	536	vikvaghrigtyleesali-----shpavaesavvgvpdaikgevpiaf
		VINVSGHR GT E E A V VVG PH KG P AF
SEQ ID NO:39	759	iqpapgrhltagadrrridelvrtekavsvpedyle--vsafpetrsgkym
SEQ ID NO:130	615	vtlcdghtfepklteelkkqirekigpiatp-dyiqnapglpkrsgkim
SEQ ID NO:131	580	vvikqgvapsdelrkelrehvrrtigpiaepaqiff-vtklpkrsgkim
		G R L E VR G P DYI V P TRSGK M
SEQ ID NO:39	808	rrflrnmml-deplgdtttlrnpveleeiaakiaewkrqrmaseeqqie
SEQ ID NO:130	664	rrvlrkiaqndhdldgmstvadpsvi-----
SEQ ID NO:131	629	rrllkavat-gaplgdvt-----
		RR LR D PLGD TT P V
SEQ ID NO:39	857	ryryfrieyhptasagklavvtvtnppvnalneraldeintivdhlarr
SEQ ID NO:130	690	-----
SEQ ID NO:131	647	-----
SEQ ID NO:39	907	qdvaaiivftgqgarsfvagadirqlleeihtveeamalpnnaahlafrkie
SEQ ID NO:130	690	-----shl-----
SEQ ID NO:131	647	-----ledetsveesk-----
		LE VEEA HL
SEQ ID NO:39	957	rmnkpciaaingvalggglefamachyrvadvyaefgqpeinrlilpgyy
SEQ ID NO:130	693	-----
SEQ ID NO:131	658	-----
SEQ ID NO:39	1007	gtqrlprllykrnngtgllralemlggrsvpadealklgldaiatgdq
SEQ ID NO:130	693	-----
SEQ ID NO:131	658	-----raye-----
		RA E
SEQ ID NO:39	1057	dslslacalaraaigadgqliessaavtqafrrhrheqldewrkdpdprfadd
SEQ ID NO:130	693	-----fshr-----
SEQ ID NO:131	662	-----
		F HR

SUBSTITUTE SHEET (RULE 26)

59/105

SEQ ID NO:39	1107	elrsiihprrieriirqahtvgrdaavhraldairygiingfeaglehea
SEQ ID NO:130	697	-----
SEQ ID NO:131	662	-----
SEQ ID NO:39	1157	klfaeavvdpnggkrgirefldrqsaplptrrplitpegeqlirdqkell
SEQ ID NO:130	697	-----
SEQ ID NO:131	662	-----
SEQ ID NO:39	1207	pvgspffpgvdripkwqyaqavirdptgaaahgdpivaekqilvpverp
SEQ ID NO:130	697	-----
SEQ ID NO:131	662	-----
SEQ ID NO:39	1257	ranqaliyvasevnndiwaitgipvsrfdchdrdwhvtgsggigliva
SEQ ID NO:130	697	-----
SEQ ID NO:131	662	-----
SEQ ID NO:39	1307	lgeearregrikvgdlvaiyagqsdlisplmgldpmaadfvigndtpdg
SEQ ID NO:130	697	-----
SEQ ID NO:131	662	-----
SEQ ID NO:39	1357	shqqfmlaqapqclpiptdmsieaagsyilnlgtiyralfttlqikagrt
SEQ ID NO:130	697	-----cl-----tiq-----
SEQ ID NO:131	662	-----sika-----
		CL T QIKA
SEQ ID NO:39	1407	ifiegaatgtgldaarsaarngrlvigmvsssrastllaagahgainrk
SEQ ID NO:130	702	-----
SEQ ID NO:131	666	-----
SEQ ID NO:39	1457	dpevadcftrvpedpsawaaweagqpllamfraqndgrladyvvshage
SEQ ID NO:130	702	-----
SEQ ID NO:131	666	-----
SEQ ID NO:39	1507	tafprsfqllgeprdghiptltfygatsgyhftflgkpgsasptemlrra
SEQ ID NO:130	702	-----
SEQ ID NO:131	666	-----
SEQ ID NO:39	1557	nlrageavliyygvgsddldvtggleaieaarqmgarivvvtvsdaqref
SEQ ID NO:130	702	-----
SEQ ID NO:131	666	-----
SEQ ID NO:39	1607	vlslgfgaalrgvvslaelkrrfgdefewprtmpplpnarqdpqglkeav
SEQ ID NO:130	702	-----
SEQ ID NO:131	666	-----emart-----
		E RT
SEQ ID NO:39	1657	rrfndlvfkplgsavgvflrsadnprgypdliieraahdalavsamlip
SEQ ID NO:130	702	-----
SEQ ID NO:131	671	-----

SUBSTITUTE SHEET (RULE 26)

60/105

SEQ ID NO:39 1707 ftgrivvyfediggrysffapqiwrqrriympaqifgthlsnayeilr
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

SEQ ID NO:39 1757 lndeisaglltitepavvpwdelpeahqamwenrhtaatyvvnhalprlg
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

SEQ ID NO:39 1807 lknrdelyeawtager
SEQ ID NO:130 702 -----
SEQ ID NO:131 671 -----

SUBSTITUTE SHEET (RULE 26)

61/105

Figure 41

SEQ ID NO:39	1	midtaplappraprsnpirdrvdweaqraaaladpgafhgaiartvihwy
SEQ ID NO:132	1	-----
SEQ ID NO:133	1	-----
SEQ ID NO:39	51	dpqhhcwirfnessqrwegldaagapvtvdypadyqpwwqafddseapf
SEQ ID NO:132	1	-----
SEQ ID NO:133	1	-----md----- D
SEQ ID NO:39	101	yrwfsaggltnacfnvdrhvmmggygdevayyfgdrwdnslnngrggppv
SEQ ID NO:132	1	-----malnn-----
SEQ ID NO:133	3	-----fnnv----- FN V LNN
SEQ ID NO:39	151	qetitrrrllvevvkaaqvlrdlgkkgdrialnmpnimpqiyyteaakr
SEQ ID NO:132	6	-----
SEQ ID NO:133	7	-----llnkddgial----- L K D IAL
SEQ ID NO:39	201	lgilytpvfvggfsdktlsdrihnagarvvitsdgayrnaqvvpkyeaytd
SEQ ID NO:132	6	-----
SEQ ID NO:133	17	-----
SEQ ID NO:39	251	qaldkyipvetaqaivaqtiatpltesqrqtiiteveaalageitvers
SEQ ID NO:132	6	-----vileks-----
SEQ ID NO:133	17	----- I E E
SEQ ID NO:39	301	dvmrgvgsalaklrdldasvqakvrtvlaqalvespprveavvvvrtgq
SEQ ID NO:132	12	-----
SEQ ID NO:133	17	-----
SEQ ID NO:39	351	eilwnegrdrwshdlldaalakilanaraagfdvhsendlilnpddqlir
SEQ ID NO:132	12	-----
SEQ ID NO:133	17	-----liin----- I N
SEQ ID NO:39	401	alyasipcepvdacypmfiiytsgstgkpkgvihvghgyvagvvhtrvs
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----
SEQ ID NO:39	451	fdaepgdtiyviadpgwitggsymltatmagrltgviaegsplfpsagry
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----
SEQ ID NO:39	501	aslierygvqifkagvtflktvmsnpqavedvrllydmhslrvatfcaepv
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----

SUBSTITUTE SHEET (RULE 26)

62/105

SEQ ID NO:39	551	spavqqfgmqimtpqyinsywatehggivwthfygnqdfplrpdahtypl
SEQ ID NO:132	12	-----
SEQ ID NO:133	21	-----rpka-----
		RP A
SEQ ID NO:39	601	pwmgdvwaetdesgttryrvadfdekgeivitapypyltrtlwgdvpg
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	651	feaylrgeiplrawkgdaerfvktywrrgpngegyiqgdfaikypdgsf
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	701	tihrpddvinvsghrmgteiegalldrqrtpdspvgncivvgaphre
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	751	kgltpvafiqpapgrhltgadrrrldelvrtekavsvpedylevsafpe
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	801	trsgkymrrflrnmmldeplgdtttlrnpevleeiaakiaewkrrqmae
SEQ ID NO:132	12	-----
SEQ ID NO:133	25	-----
SEQ ID NO:39	851	eqqieryryfrieypptasagklavvtvtnpp-vnalneraldelnti
SEQ ID NO:132	12	-----gkvavvtinrpkalnalsdtlkemdyv
SEQ ID NO:133	25	-----lnalnyetlkeldsv
		GK AVVT P NALN L EL
SEQ ID NO:39	900	vdhlarrqdvaaivftggarsfvagadirqlleeihtve-eamalpnna
SEQ ID NO:132	40	igeiendsevlaviltgageksfvagadisem-kemntiegrkfgilgnk
SEQ ID NO:133	40	ldivendkeikvliitgsgektfvagadiaemsn--mtpl-eakkslyg
		D V A TG G SFVAGADI E T E EA N
SEQ ID NO:39	949	hlafrkiermnkpciaaingvalggglefamachyrvadvyaeffgqpein
SEQ ID NO:132	89	--vfrlellekpviaavngfalgggceiamsedirassnarffgqpevg
SEQ ID NO:133	87	qkvfrkiemlskpviaavngfalgggcelsmacdiriasknakfgqpevg
		FRKIE KP IAA NG ALGGG E ANAC R A A FGQPE
SEQ ID NO:39	999	lrlpgygggtqrlprllykrnngtgllralemlggrsvpadealkgli
SEQ ID NO:132	137	lgitpgfggtqrlsrly-----gmgmakqliftaqlikadealriglv
SEQ ID NO:133	137	lgiipgfsgtqrlprli-----gtakakeliftgdmnsdeaykigli
		L PG GGTQRLPRL G A E I G ADEALK GLI
SEQ ID NO:39	1049	daiatgdqdsllacalaraaigadgqliesaaavtqafhrheqldewrk
SEQ ID NO:132	180	n-----
SEQ ID NO:133	180	skvv-----
SEQ ID NO:39	1099	pdrfaddelrsiliahprieriirqahtvgrdaavhraldairygiihgf
SEQ ID NO:132	181	-----
SEQ ID NO:133	184	-----elsdli-----
		EL I

SUBSTITUTE SHEET (RULE 26)

63/105

SEQ ID NO:39	1149	eagleheaklfaeavvdpnggkrgirefldrqsaplptrrplitpeqeql
SEQ ID NO:132	181	-----kvveps-----el
SEQ ID NO:133	190	-----eeakklak-----
		EAK A VV P L
SEQ ID NO:39	1199	lrdqkellpvgsppffpgvdripkwqyaqavirdptgaaahgdpivaekq
SEQ ID NO:132	189	mntakei-----
SEQ ID NO:133	198	-----kmmsskq
		KE Q
SEQ ID NO:39	1249	liipverpranqaliyvlasenvfndiwaitgipvsrfdehdrdwhvtgs
SEQ ID NO:132	196	-----ank-----ivsnapva-----
SEQ ID NO:133	205	i-----
		I AN PV
SEQ ID NO:39	1299	ggiglivalgeearregrikvgdlvaiysggsdillsplmgldpmaadfvi
SEQ ID NO:132	207	-----vklskgainrgm-----
SEQ ID NO:133	206	-----aislakeainkg-----
		V L EA G
SEQ ID NO:39	1349	qgndtpdgshqqfmlaqapqccliptdmsieaagsyilnlgtiyralftt
SEQ ID NO:132	219	-----qc-didtalafesea-----fyecfst
SEQ ID NO:133	218	-----metdld-----
		QC I TD E F T
SEQ ID NO:39	1399	lqikagrtifiegaatgtgldaarearngrlvigmvssssrastllaag
SEQ ID NO:132	240	edqkdamtatie-----
SEQ ID NO:133	224	-----tgntieaekfsl-----
		K T FIE TG A
SEQ ID NO:39	1449	ahgainrkdpevadcftrvpdpasawaaweagqpllamfraqndgrlad
SEQ ID NO:132	252	-----
SEQ ID NO:133	236	-----cft-----
		CFT
SEQ ID NO:39	1499	yvvshagetafprsfqlgeprdgthiptltfygatsgyhftflgkpgsas
SEQ ID NO:132	252	-----
SEQ ID NO:133	239	-----
SEQ ID NO:39	1549	ptemlrranlrageavliyyvggsddlvdtggleaieaarqmgarivvvt
SEQ ID NO:132	252	-----
SEQ ID NO:133	239	-----
SEQ ID NO:39	1599	vsdaqrefvlslgfgaalrgvvsiaelkrfrgdefewprtmpplpnarqd
SEQ ID NO:132	252	-----krk-----
SEQ ID NO:133	239	-----tddqke-----gmiafse-kr-----
		D Q E G E KR
SEQ ID NO:39	1649	pqglkeavrrfndlvfkplgsavgvflrsadnprgypdliieraahdala
SEQ ID NO:132	255	-----le-----
SEQ ID NO:133	254	-----
		IE
SEQ ID NO:39	1699	vsamlikpftgrivfyfediggrrysfapqiwwrqrriymptaqifgthl
SEQ ID NO:132	257	-----
SEQ ID NO:133	254	-----apk-----fgk-----
		AP FG

SUBSTITUTE SHEET (RULE 26)

64/105

SEQ ID NO:39 1749 snayeilrlndeisaglltitepavvpwdelpeahqamwenrhtaatyvv
SEQ ID NO:132 257 -----
SEQ ID NO:133 260 -----

SEQ ID NO:39 1799 nhalprlgknrdelyeawtager
SEQ ID NO:132 257 -----gfnr-----
SEQ ID NO:133 260 -----

G KNR

SUBSTITUTE SHEET (RULE 26)

65/105

Figure 42

SEQ ID NO:39	1	midtaplappraprsnpirdrvdweagraaaladpgafhgaiartvihwy
SEQ ID NO:134	1	-----
SEQ ID NO:135	1	-----
SEQ ID NO:39	51	dpqhhcwirfnessqrweglfaatgapvtvdypadyqpwwqafddseapf
SEQ ID NO:134	1	-----maasaap-----
SEQ ID NO:135	1	-----
AA AP		
SEQ ID NO:39	101	yrwfsqgltncfnevdrhvmgygdevayyfeqdrwdnslnnrggpgvv
SEQ ID NO:134	8	-----
SEQ ID NO:135	1	-----
SEQ ID NO:39	151	qetitrllvevkaaqvlrdlgkkgdrialnmpnimpqiyyteaakr
SEQ ID NO:134	8	-----
SEQ ID NO:135	1	-----
SEQ ID NO:39	201	lgilytpvfggfsdktlsdrihnagarvitsdgayrnaqvpykeaytd
SEQ ID NO:134	8	-----awtg-----
SEQ ID NO:135	1	-----
A T		
SEQ ID NO:39	251	qaldkiyvetaqaivaqtlatpltesqrqtiitevesalageitvers
SEQ ID NO:134	12	q-----taeak
SEQ ID NO:135	1	-----mtiqtleltalkd-----
Q QTL T L T E		
SEQ ID NO:39	301	dvargvgasalaklrldasvqakvrtvlaqalvespprveavvvvzhtgq
SEQ ID NO:134	18	d-----
SEQ ID NO:135	14	-----
D		
SEQ ID NO:39	351	eilwnegrdrwshdlldaalakilanaraagfdvhsendlilnpddqlir
SEQ ID NO:134	19	-----
SEQ ID NO:135	14	-----
SEQ ID NO:39	401	alyasipcepvdaeypmfiiytsgstgkpkgvihvhggyvagvvhtlrva
SEQ ID NO:134	19	-----
SEQ ID NO:135	14	-----
SEQ ID NO:39	451	fdaepgdtiyviadpgwitgqsymltatmagrltgviaegsplfpsagry
SEQ ID NO:134	19	-----
SEQ ID NO:135	14	-----
SEQ ID NO:39	501	asiierygvqifkagvtflktvmsnpqnvdrlydmhslrvatfcaepv
SEQ ID NO:134	19	-----lyei-----
SEQ ID NO:135	14	-----lyei-----
LY		

SUBSTITUTE SHEET (RULE 26)

66/105

SEQ ID NO:39	551	spavqqfgmqimtpqyinsywatehggivwthfygnqdfplrpdahtyp1
SEQ ID NO:134	23	-----
SEQ ID NO:135	18	-----
SEQ ID NO:39	601	pwvmgdvwaetdesgttryrvadfddekgeivitapypyltrtlwgdvpg
SEQ ID NO:134	23	-----
SEQ ID NO:135	18	-----
SEQ ID NO:39	651	feaylrgeiplrawkgdaerfvktywrrgpngegyiqgdfaikypdgsf
SEQ ID NO:134	23	-----geip-----
SEQ ID NO:135	18	-----geip-----
		GEIP
SEQ ID NO:39	701	tlhgrpddvinvsghrmgteiegalldrqi tpdspvgncivvgaphre
SEQ ID NO:134	27	-----
SEQ ID NO:135	22	-----
SEQ ID NO:39	751	kg1tpvafiqpapgrhltgadrrrldelvrtekavsvpedyievsafpe
SEQ ID NO:134	27	-----
SEQ ID NO:135	22	-----pafhv-----pk
		P H P
SEQ ID NO:39	801	trsgkymrrflrnmmldeplgdtttlrnpevl e eiaakiaewkrrqmae
SEQ ID NO:134	27	-----plg-----hvpakmyawairr-----
SEQ ID NO:135	29	t-----myawsirk-----
		T PLG AK W R
SEQ ID NO:39	851	eqqlieryryfrieypptasagklavvtvtnppvnalneral delntiv
SEQ ID NO:134	43	-----erh-----
SEQ ID NO:135	38	-----
		ER
SEQ ID NO:39	901	dhlarrqdvaaivftgggarfvagadirqlleeihtveeamalpnahl
SEQ ID NO:134	46	-----
SEQ ID NO:135	38	-----
SEQ ID NO:39	951	afrkiermnkpciaaingvalggglefamachyrvadvyafgqpeinlr
SEQ ID NO:134	46	-----gppe-----
SEQ ID NO:135	38	-----erhgkp-----
		ER KP G PE
SEQ ID NO:39	1001	llpgygggtqrlprllykrnngtgllralemlggrsvpadealklglda
SEQ ID NO:134	50	-----
SEQ ID NO:135	44	-----
SEQ ID NO:39	1051	iatgdqds1slacalaraaigadgqliesaaavtqafrrheql dewrkpd
SEQ ID NO:134	50	-----
SEQ ID NO:135	44	-----tqamq-----
		TOA
SEQ ID NO:39	1101	prfaddelrsiihprieriirqahtvgrdaavhraldairygiibgfea
SEQ ID NO:134	50	-----qsh-----
SEQ ID NO:135	49	-----
		Q R

SUBSTITUTE SHEET (RULE 26)

67/105

SEQ ID NO:39	1151	gleheaklfaeavvdpnggkrgirefldrqsapltrrplitpeqeqllr
SEQ ID NO:134	53	-----
SEQ ID NO:135	49	-----
SEQ ID NO:39	1201	dqkellpvgsppffpgvdripkwqyaqavirdpdtgaaahgdpivaekqii
SEQ ID NO:134	53	-qlevlpv-----wei-----gd-----
SEQ ID NO:135	49	-----vevvtweige-----
		Q E LPV V P W GD
SEQ ID NO:39	1251	vpverpranqaliyvllasevnfnidwaitgipvsrfdehdrdwhvtgsgg
SEQ ID NO:134	65	-----devlvymaagvnyngvwaglgpispfdvhkgeyhiagsda
SEQ ID NO:135	60	-----devlvymaagvnyngvwaaigepispldghkqpfhiagsda
		L Y V A V N N W A G P S F D H H G S
SEQ ID NO:39	1301	iglivalgeearregrikgvdlvaiysggsdlisp-lmgldpm-aadfiv-
SEQ ID NO:134	107	sgivwkvgakvk---rwkvgdevivhcnqddgddeecnggdp-fsptqr
SEQ ID NO:135	102	sgivwkvgakvk---rwklgdevvihcnqddgddeecnggdp-fsptqr
		G G R K V G D V I Q D G D F M
SEQ ID NO:39	1348	iqgndtpdgshqqfmlaqapqcipiptdmsieaagseyilnltiyralf-
SEQ ID NO:134	153	iwgyetgdgsfaqfcrvqsrqlmarphkltweeaacytitlatayrmlfg
SEQ ID NO:135	148	iwgyetpdgsfaqfcrvqsrqlprkhlteesacytitlatayrmlfg
		I G T P D G S Q F Q Q L P P E A Y L L T Y R L F
SEQ ID NO:39	1397	-ttlqikagrttiefiegaatgtgldaarsaarnlrvigmvssasrastll
SEQ ID NO:134	203	haphtvrpgqnvliwgasggigvfgvqlcaasganaiavisdeskrdyvm
SEQ ID NO:135	198	hkphelkpgqnvliwgasggigvfatqlaavaganaigvvsedkrefvl
		K G I G A G G A A A G I G V S S S L
SEQ ID NO:39	1446	aagahgainrkdpevadcftrvpdpdpasawaawaagqpllamfraqngr
SEQ ID NO:134	253	slgakgvnrkd---fdc---w-----
SEQ ID NO:135	248	smgakavlnrge---fncwgqlpk-----
		GA G I N R K D D C P
SEQ ID NO:39	1496	ladyvvhshagetafprsfqllgeprdgthiptltfygatsgyhftflgkpg
SEQ ID NO:134	269	-----gqlptv-----
SEQ ID NO:135	269	-----vngpef-----
		G P T G F
SEQ ID NO:39	1546	sasptemlrranlrageavliyygvgsddldvdtggglealeaarqmgariv
SEQ ID NO:134	275	-----
SEQ ID NO:135	275	-----
SEQ ID NO:39	1596	vvtvsdaqrefvlsigfgaalrgvvsaelkrrfgdefewprtmpplpna
SEQ ID NO:134	275	-----ns-----
SEQ ID NO:135	275	-----ndymke-----srkfgkai-wqit-----
		D E R F G W T N
SEQ ID NO:39	1646	rqdpqglkeavrrfndlvfkplgsavgvflrsadnprgydpdlieraahd
SEQ ID NO:134	277	peyntwlkea-rkfgkaiwditgkndv-----divfehpgae
SEQ ID NO:135	293	-----gnkdv-----dmvfehpgae
		GLKEA R F G V D E
SEQ ID NO:39	1696	alavsamlikpftgrivfyfediggrrysfapqiwvrqriymptaqifg
SEQ ID NO:134	314	tfpvstlvakr-ggmivfcagttgfnitfdaryvwmrqkriq-----g
SEQ ID NO:135	308	tfpvsvflvkr-ggmivvicagttgfnitmdarfllwmrqkrvq-----g
		V S L K G I V G F A W R Q R I G

SUBSTITUTE SHEET (RULE 26)

68/105

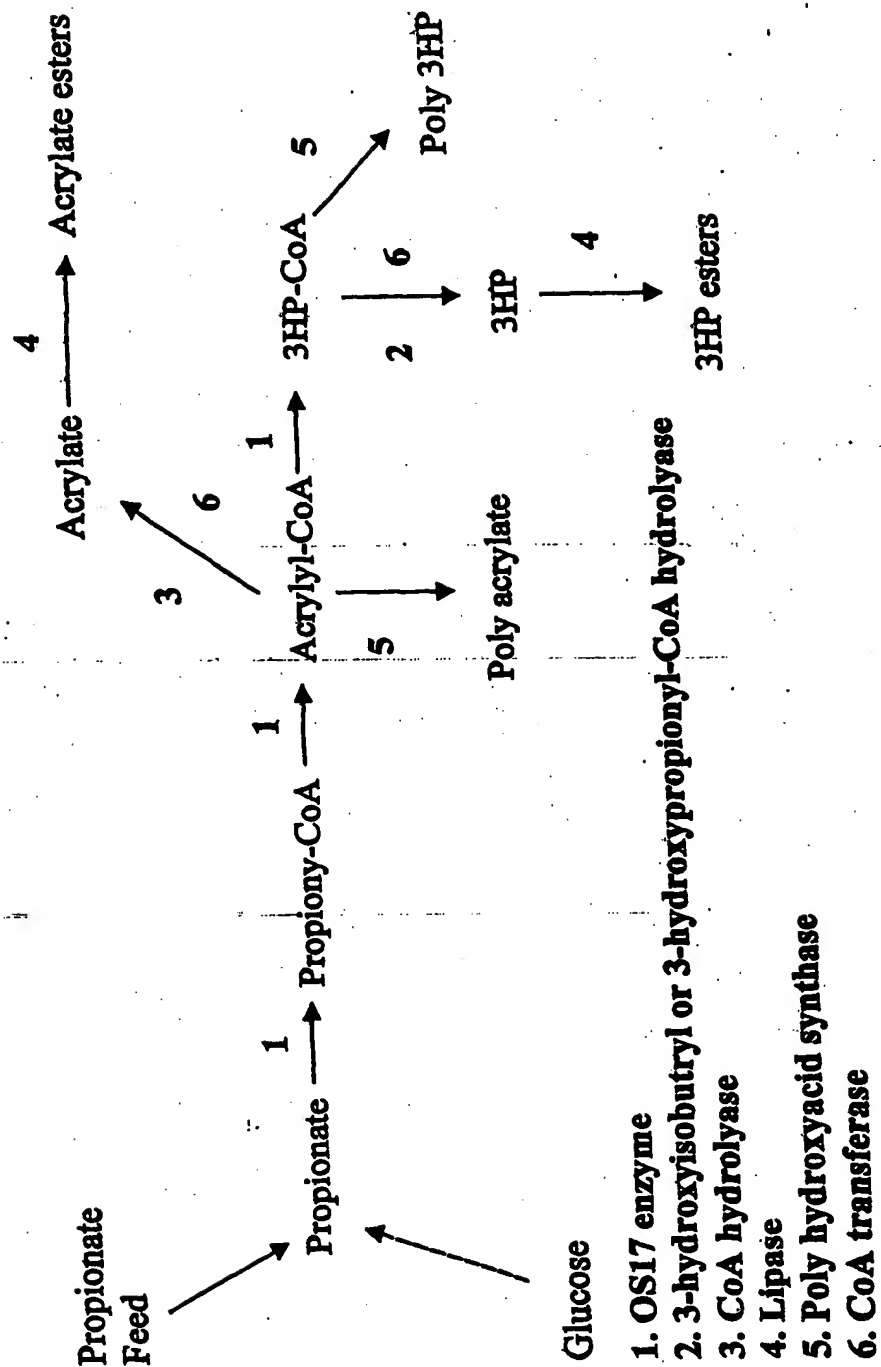
SEQ ID NO:39 1746 thlsnayeilrlndeisaglltitepavvpwdelpeahqamwenrhta
 SEQ ID NO:134 356 shfahlkqasaangfvmddrpdcmsevpwdkipaahkmmwknghppgn
 SEQ ID NO:135 350 shfanlmqasaanqlvidrrvdpcisevpwdqipaahkmlanghlpn
 H N N V PWD P AH MW N H

SEQ ID NO:39 1796 yvvnhalprlgikndelyeawtager
 SEQ ID NO:134 406 mavlvnstraglrtdvedvieagplkam
 SEQ ID NO:135 400 mavlvcaqrpglrtdfeevqelsgap--
 V R GL E EA A

SUBSTITUTE SHEET (RULE 26)

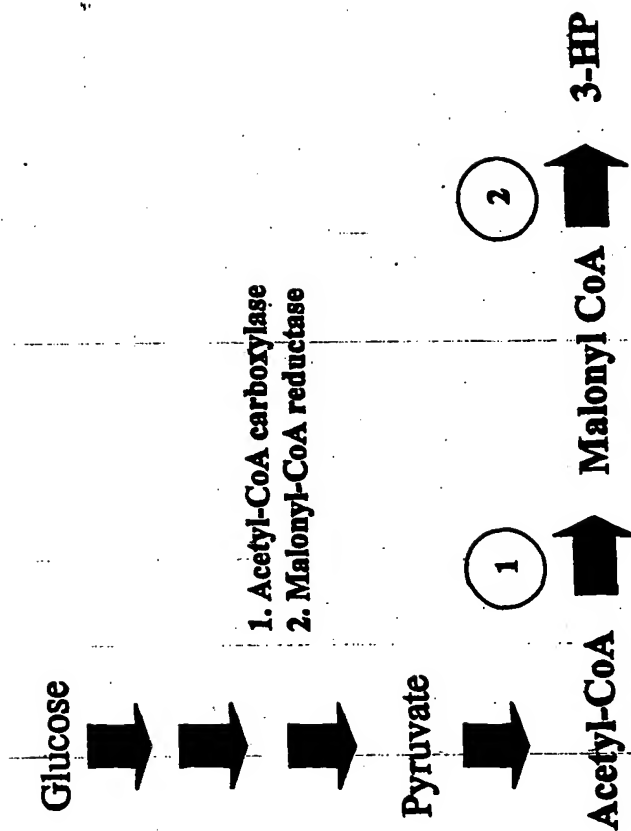
69/105

Figure 43



SUBSTITUTE SHEET (RULE 26)

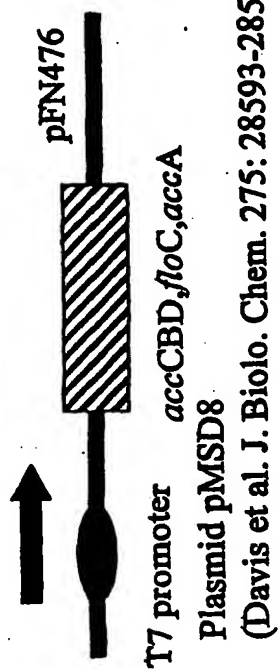
70/105

Figure 44

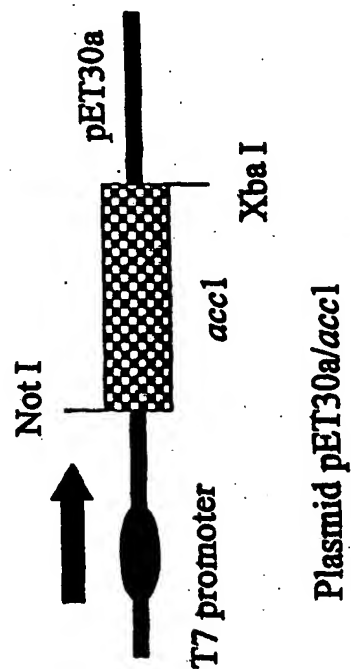
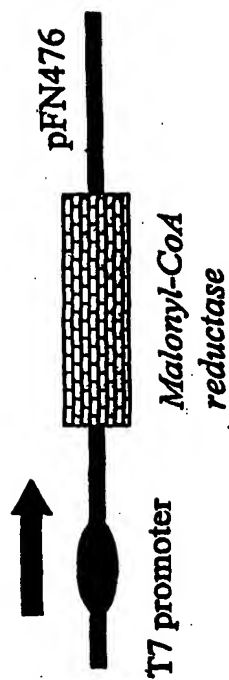
71/105

Figure 45

Acetyl-CoA carboxylase constructs

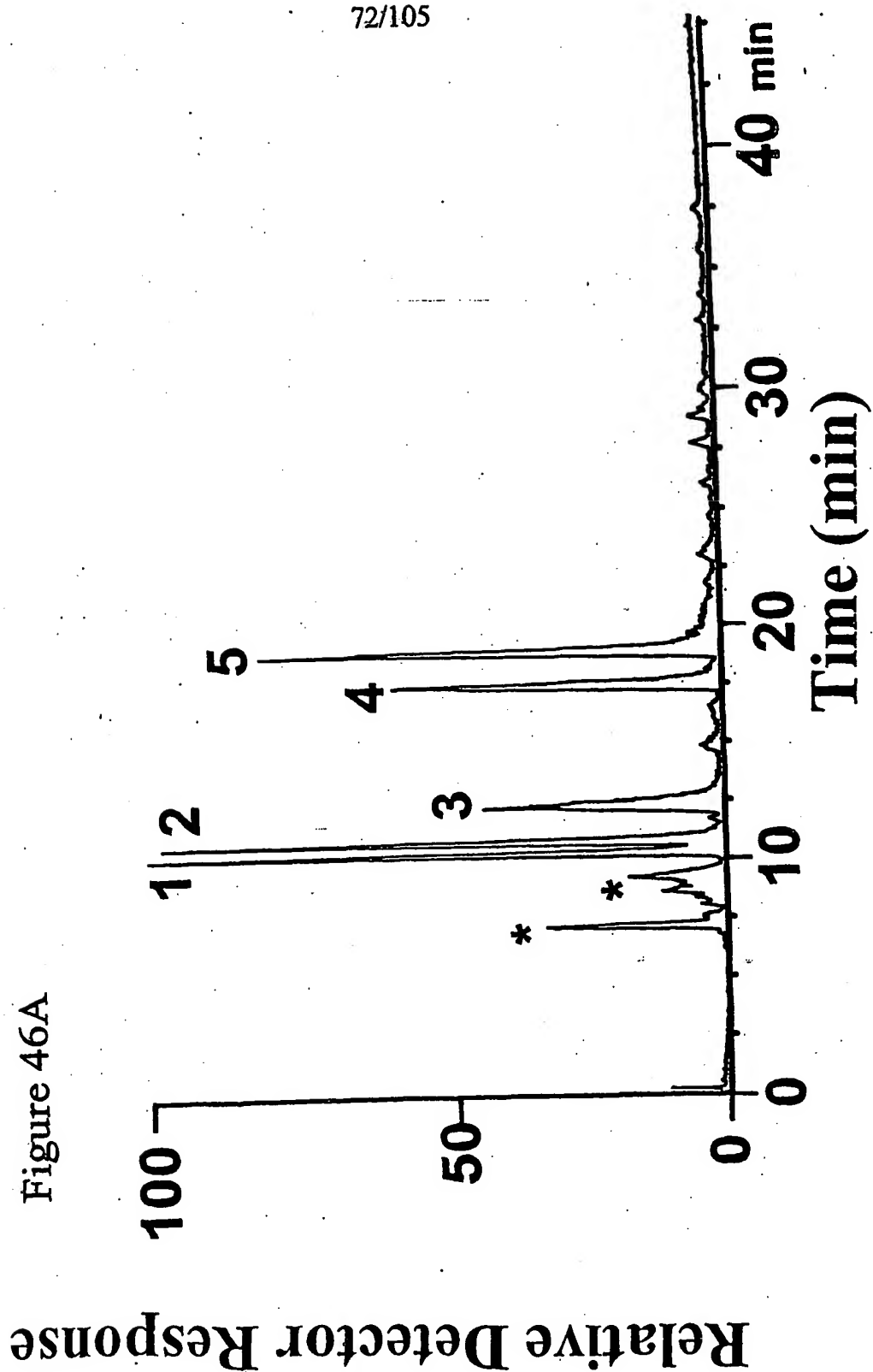


Malonyl-CoA reductase constructs



SUBSTITUTE SHEET (RULE 26)

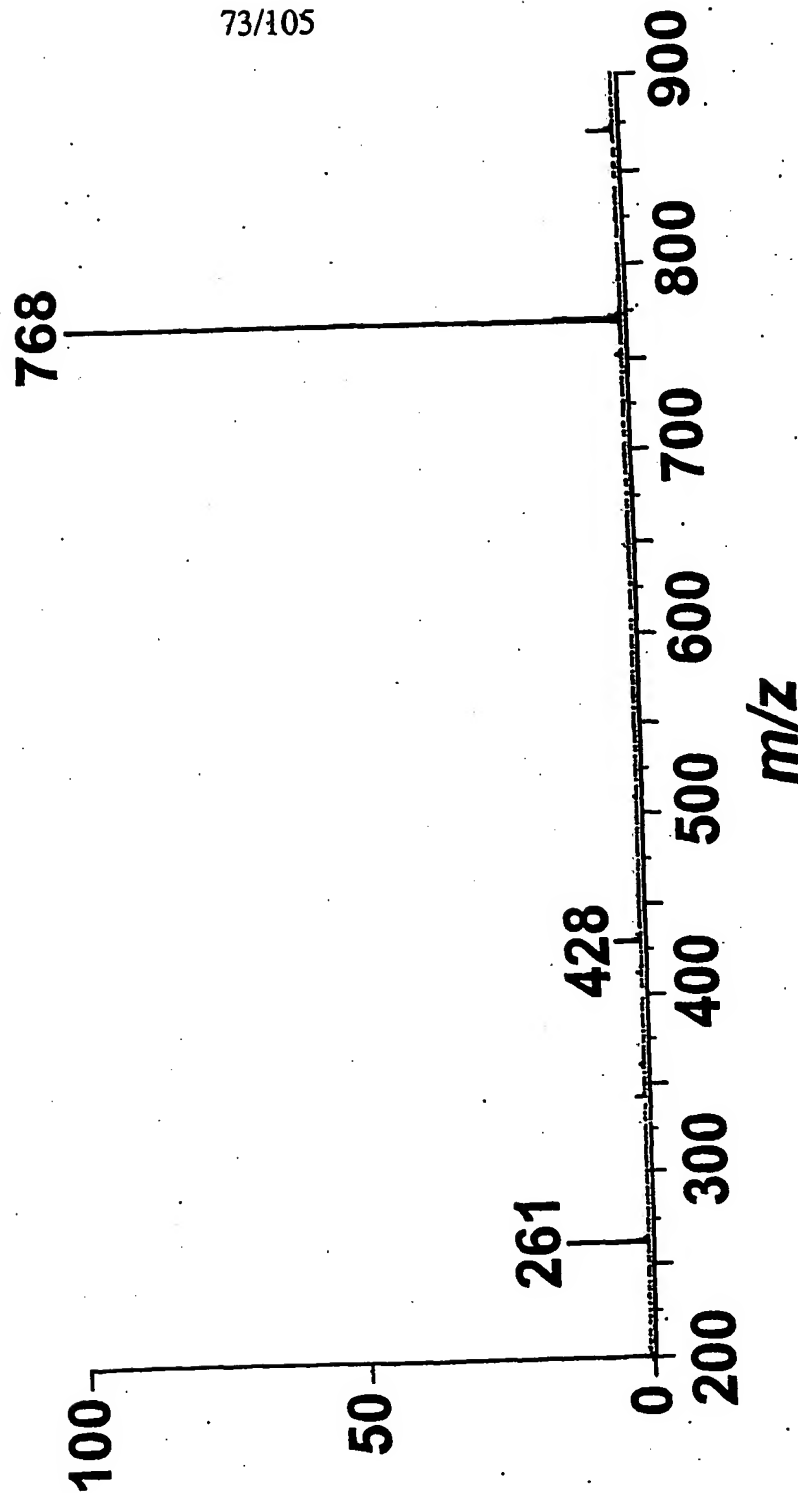
72/105



73/105

Figure 46B

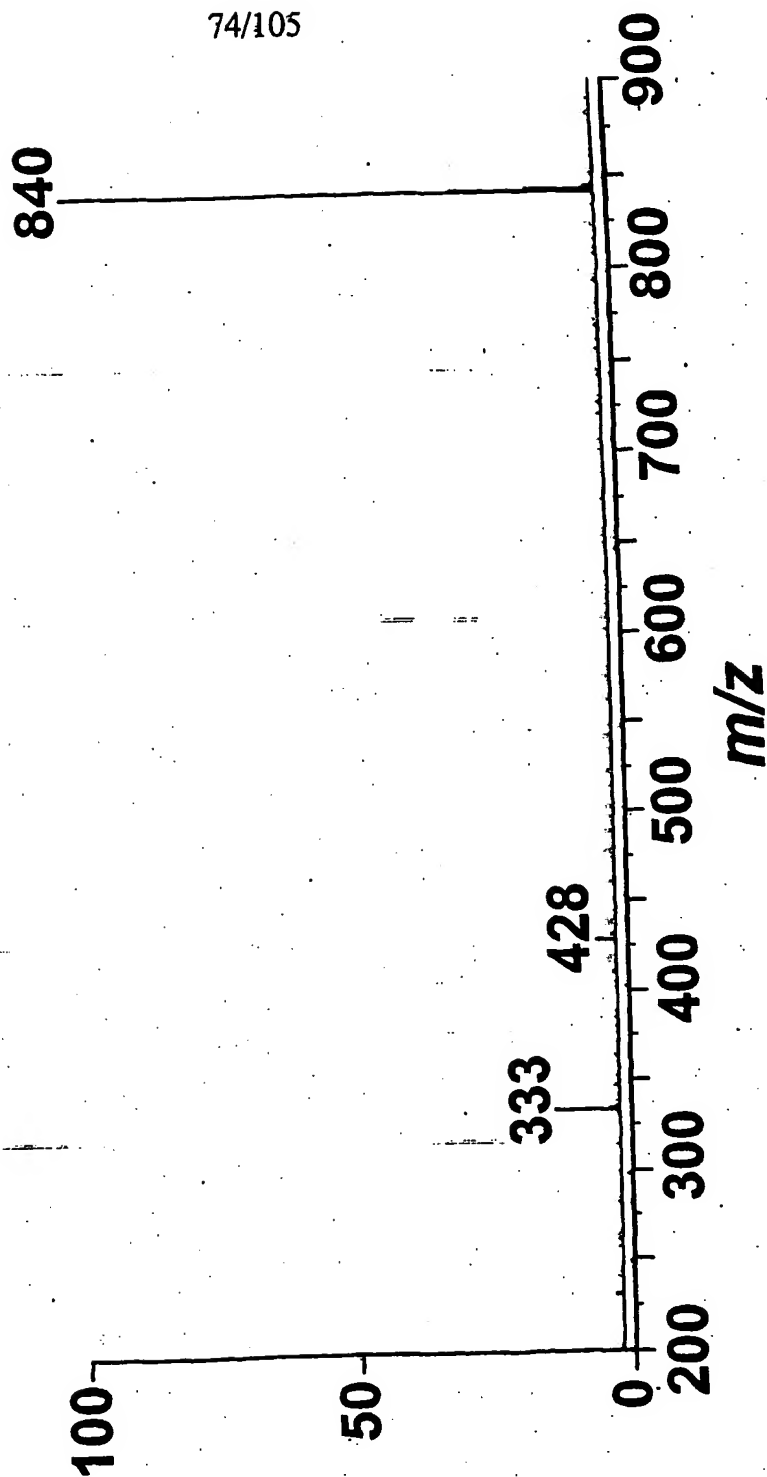
Relative Detector Response



SUBSTITUTE SHEET (RULE 26)

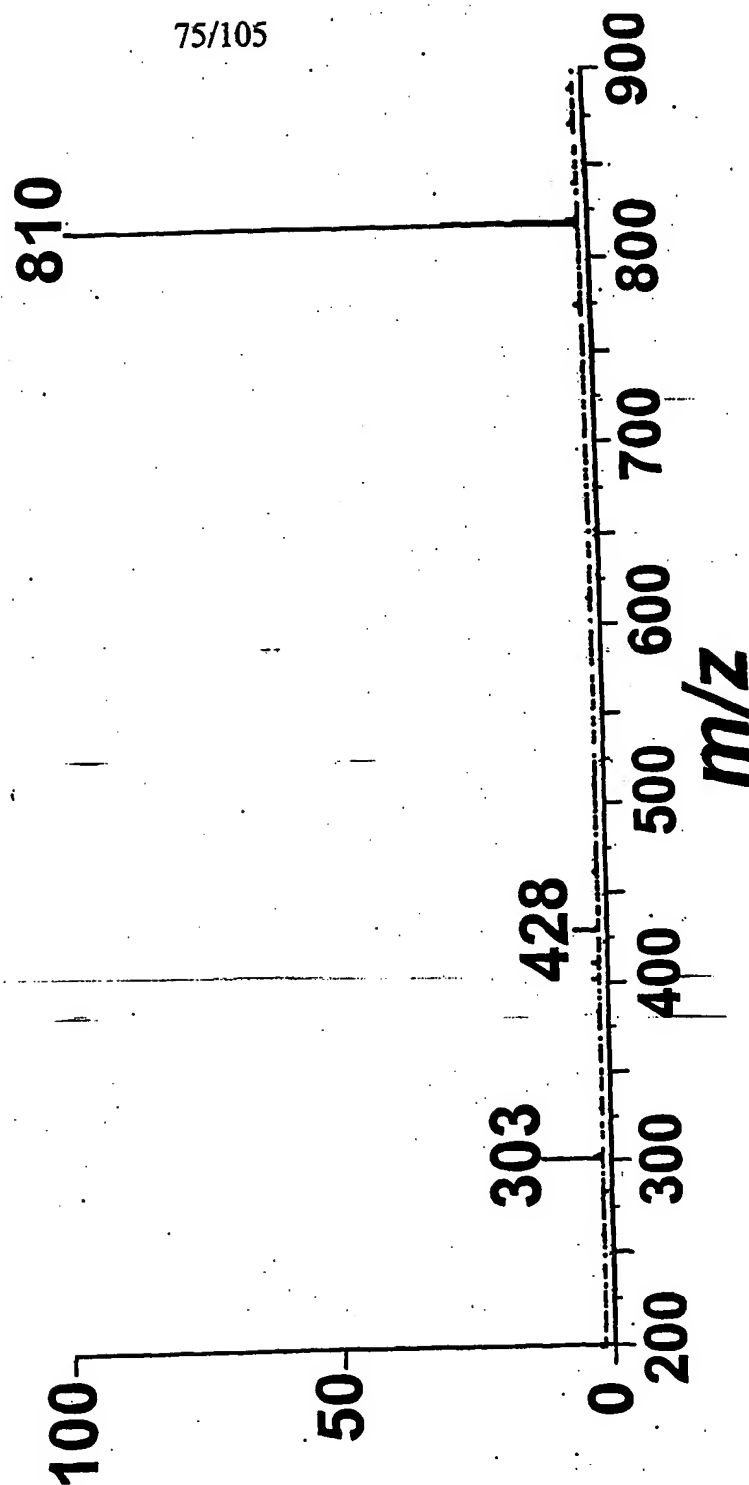
Relative Detector Response

Figure 46C



Relative Detector Response

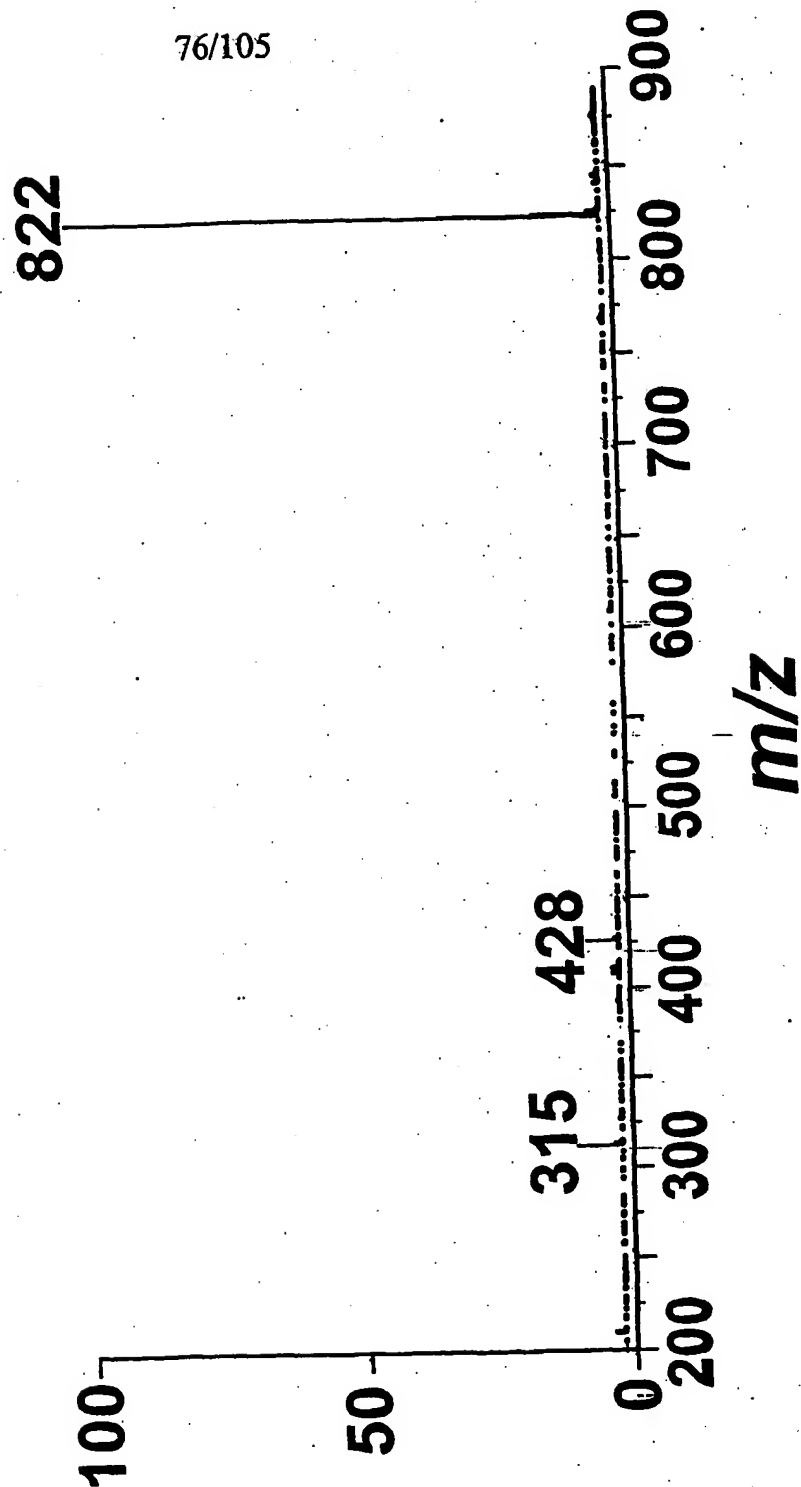
Figure 46D



SUBSTITUTE SHEET (RULE 26)

Relative Detector Response

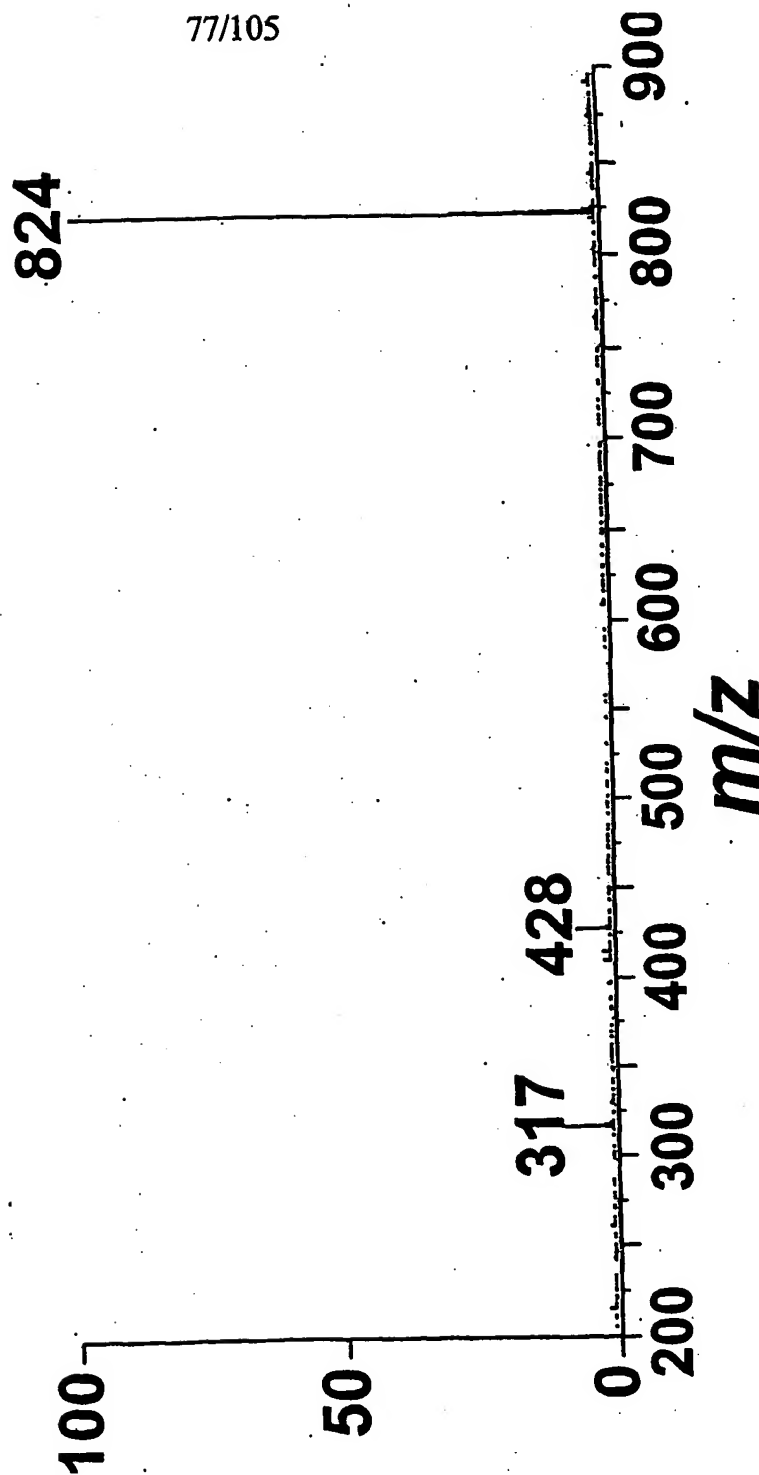
Figure 46E



SUBSTITUTE SHEET (RULE 26)

Relative Detector Response

Figure 46F



SUBSTITUTE SHEET (RULE 26)

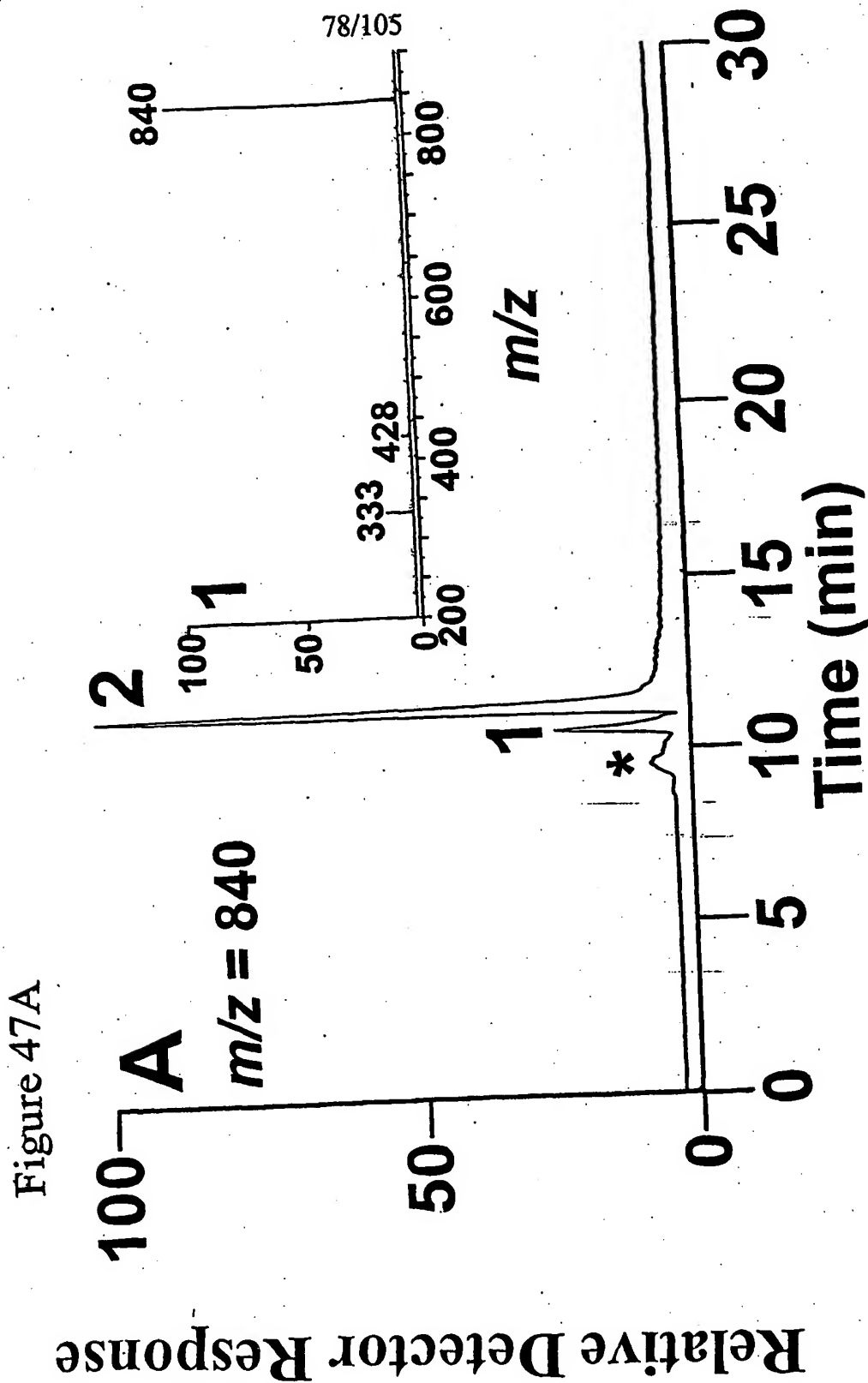
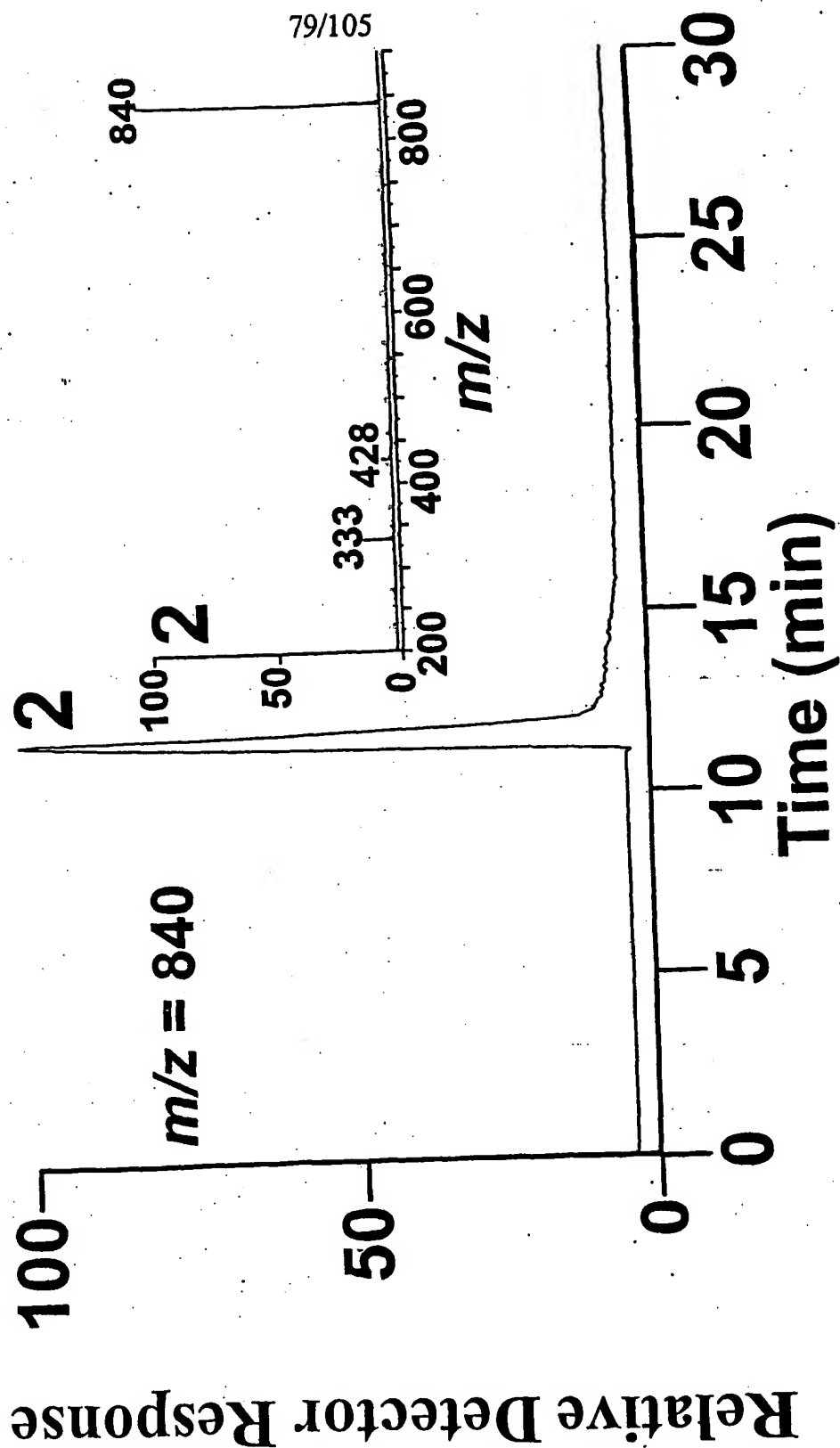


Figure 47B



SUBSTITUTE SHEET (RULE 26)

Figure 48A

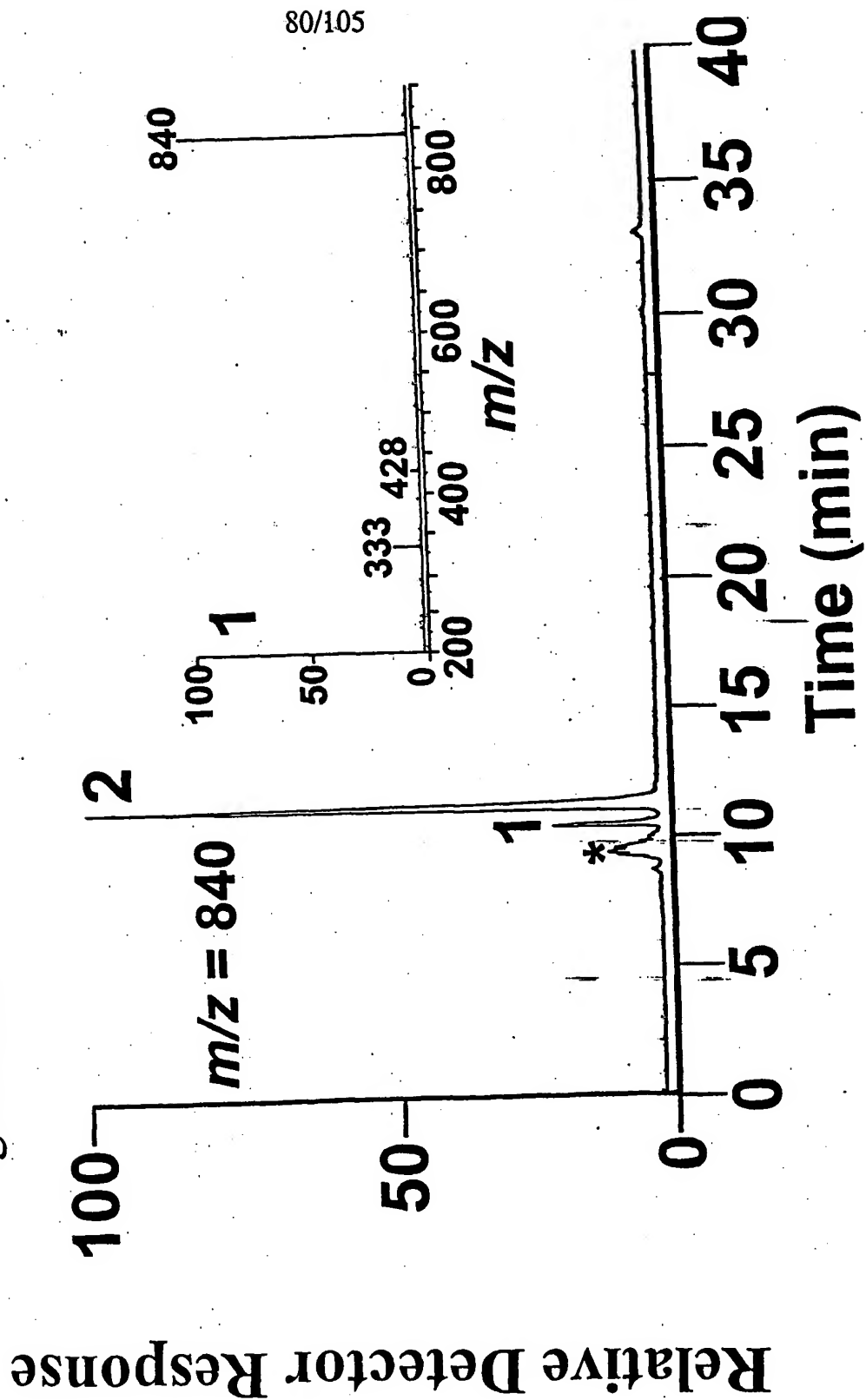


Figure 48B

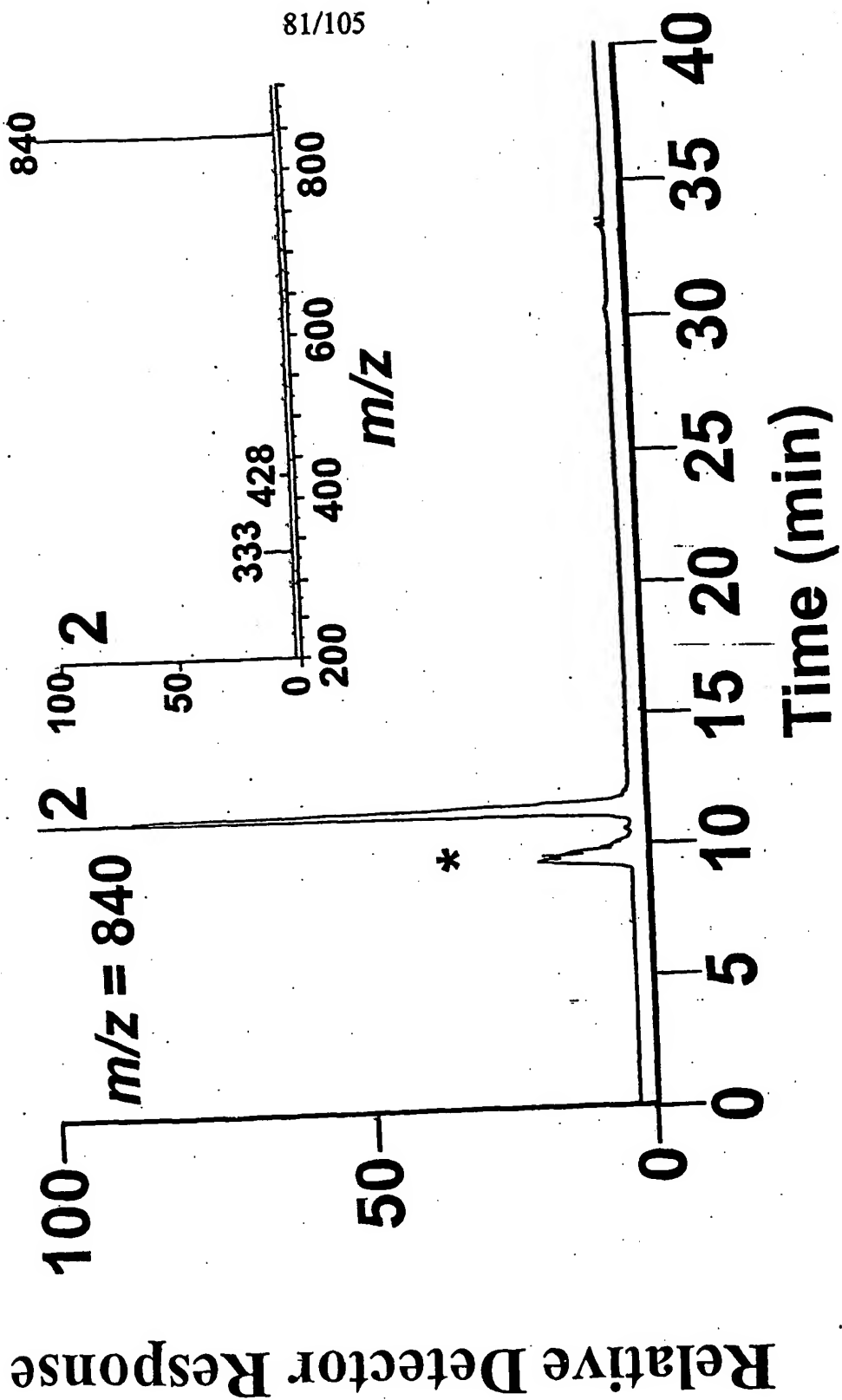


Figure 49

SUBSTITUTE SHEET (RULE 26)

83/105

GAACGCACCCCTACCGGTGGCGAACTCTTCGGCTTGCCCTCACCGGAACGGCTGGCGGAG
CTGGTCGGAAGCACGGTCTATCTGATAGGTGAACATCTGACTGAACACCTTAACCTGCTT
GCCCCGTGCGTACCTCGAACGTTACGGGGCACGTCAGGTAGTGATGATTGTTGAGACAGAA
ACCGGGGCAGAGACAATGCGTCGCTTGCTCCACGATCACGTCGAGGCTGGTGGCTGATG
ACTATTGTGGCCGGTGATCAGATCGAAGCCGCTATCGACCAGGCTATCACTCGCTACGGT
CGCCCAGGGCCGGTCGTCTGTACCCCTTCCGGCCACTGCCGACGGTACCACTGGTGGG
CGTAAAGACAGTGACTGGAGCACAGTGTGAGTGAGGCTGAATTTGCCGAGTTGTGCGAA
CACCAGCTCACCCACCATTTCCGGGTAGCGCGCAAGATTGCCCTGAGTGATGGTGCCAGT
CTCGGCTGGTCACTCCCAGAACTACGGCTACCTCAACTACCGAGCAATTTGCTCTGGCT
AACTTCATCAAAACGACCTTACGCTTTTACGGCTACGATTGGTGTGCGAGAGCGAAAGA
ACTGCTCAGCGCATTCTGATCAATCAAGTCGATCTGACCCGGCGTGCGCGTGCCGAAGAG
CCGCGTGATCCGCACGAGCGTCAACAAGAACTGGAACGTTTTATCGAGGCAGTCTTGCTG
GTCACGACCACTCCCGCTGAAGCCGATACCCGTTACGCCGGGCGGATTATCGCGGA
CGGGCGATTACCGTGTA (SEQ ID NO:140)

SUBSTITUTE SHEET (RULE 26)

84/105

Figure 50

MATGESMSGTGRLAGKIALITGGAGNIGSELTRRFLAEGATVIISGRNRAKLTAALERMQ
AEAGVPAKRIDLEVMGSDPVAVRAGIEAIVARHGQIDILVNNAGSAGAQRRLAEIPLTE
AELGPAGAEETLHASIANLLGMGWHLMRIAPHMPVGSVINSTIFSRAEYYGRI PYVTP
KAALNALSQLAARELGARGIRVNTIFPGPIESDRITVFQRM DQLKGRPEGDTAHHFLNT
MRLCRANDQGALERRFPSVGDVADA AVFLASAESAALSGETIEVTHGMELPACSETSLA
RTDLRTIDASGRITLICAGDQIEEVMALTGMLRTCGSEVIIGFRSAAALAQFEQAVNESR
RLAGADFTPPIALPLDPRDPATIDAVFDWAGENTGGIHA AVILPATSHEPAPCVIEVDDE
RVLNFLADEITGTIVIASRLARYWQSQR LTPGARARGPRVIFLSNGADQNGNVYGRIQSA
AIGQLIRVWRHEAELDYQRASAAGDHVLPFVWANQIVRFANRSLEGLEFACAWTAQLLHS
QRHINEITLNI PANISATTGARSASVGWAESLIGLHLGKVALITGGSAGIGGOIGRLLAL
SGARVMLAARDRHKLEQMAMIQSELA EVGYTDVEDRVHIA PGCDVSSEAQLADLVERTL
SAFGTVDYLINNAGIAGVEEMVIDMPVEGW RHTLFANLISNYSIMRKLAFLMKKGSGYI
LNVSSYFGGEKDAI PYPNRADYAVSKAGQ RAMAEVFARFLGPEIQINAIAPGPVEGDRL
RGTGERPGLFARRARLILENKRLNELHAALIAAARTDERSMHELVELLLENDVAALEQNP
AAPTALRELARRFRSEGDPAASSSSALLNRSIAAKLLARLHNGGYVLPADIFANLPNPPD
PFFTQAQIDREARKVRDGMMLYLQRMPT EFDVAMATVYYLADRNVSGETFHPSGGLRY
ERTPTGGELFGLPSPERLAELVGSTVYLI GEHLTEHLNLLARAYLERYGARQVVMIVETE
TGAETMRRLLDHVEAGRLMTIVAGDQIEAAIDQAITRYGRPGPVVCTPFRPLPTVPLVG
RKDSDWSTVLSEAEFAELCEHQLTHHFRVARKIALSDGASLALVTPETTATSTTEQFALA
NFIKTTLHAFTATIGVESERTAQRILINQVDLTRRARAEEPRDPHERQOELERFIEAVLL
VTAPLPPEADTRYAGRIHRGRAITV (SEQ ID NO:141)

SUBSTITUTE SHEET (RULE 26)

85/105

Figure 51

TCTTTCTGGCCAGTGCCGAATCCGCCGCTCTCTCCGGTGAGACGATTGAGGTTACGCACG
GAATGGAGTTGCCGGCCTGCAGTGAGACCAGCCTGCTGGCCCGTACTGATCTGCGCACGA
TTGATGCCAGTGGCCGCACGACGCTCATCTGCGCCGGCGACCAGATTGAAGAGGTGATGG
CGCTCACCAGGTATGTTGCGTACCTGTGGGAGTGAAGTGATCATCGGCTTCCGTTCCGGCTG
CGGCGCTGGCCAGTTCGAGCAGGCAGTCAATGAGAGTCGGCGGCTGGCCGGGCGCAGACT
TTACGCCTCCCATTCGCTTGCCACTCGATCCACGCG (SEQ ID NO:142)

SUBSTITUTE SHEET (RULE 26)

86/105

Figure 52

SEQ ID NO:141	1	matgesmsgtgrlagkialitggagnigseltrrflaegatvliisgrnra
SEQ ID NO:143	1	-----mfankvvlvtggssgigaatveafvkegasvafvgrnqa
SEQ ID NO:144	1	-----mrlegkvclitgaasgigkattllfaegatviagdiske
SEQ ID NO:145	1	-----mekf-----
SEQ ID NO:146	1	-----mrllhkrtltvggsdgiglaiaaeflsegadvlivgrdaa
SEQ ID NO:147	1	-----
SEQ ID NO:141	51	klatalaermqa--e-agvpakridlevmdgsdpavragieaivarhggqi
SEQ ID NO:143	40	klkevesrcqq--hganilaikadv-----skdeeakiivqqtvdkgfkl
SEQ ID NO:144	41	nldslvk--ea--e--glp-----gkv
SEQ ID NO:145	1	-----
SEQ ID NO:146	5	-----
SEQ ID NO:147	41	kleaarqklaalgq--aga----vetssadlatslgvatvveqvketgrpl
SEQ ID NO:141	98	dilvnnagsagaqrriaeiplteaelgpggaestlihasianllmgwhlmr
SEQ ID NO:143	83	dvlvnnagil----rfasv--leptliqtfdetmntnlrpv-----lits
SEQ ID NO:144	57	d-----
SEQ ID NO:145	1	-----
SEQ ID NO:146	5	-----
SEQ ID NO:147	86	dipinnagvadl-----vpfasv-----seaqfghsfalnvaaffltq
SEQ ID NO:141	148	laaphm-pvgasvinvtifgr--aeyygrlp--yvtpkaaalnalsqlaar
SEQ ID NO:143	123	laiphliatksivnvssilativripglm--ysvskaamdhtklaal
SEQ ID NO:144	58	-----p--yv-----lnv-----
SEQ ID NO:145	1	-----
SEQ ID NO:146	5	-----php-p-----
SEQ ID NO:147	125	gllphf-gagasiinissyfar--kmpkrpsvyslskgalnsltrslaf
SEQ ID NO:141	194	elgargirvntifpgpiesdrirtvfqrmddqkgrpegdtahhflntmr1
SEQ ID NO:143	171	elapsgvirvnsvnpgpv-----
SEQ ID NO:144	64	-----tdr-----
SEQ ID NO:145	1	-----mnpmdrqtgegqepqh-----
SEQ ID NO:146	9	-----
SEQ ID NO:147	173	elgprgirvnaiapgtvdt-----
SEQ ID NO:141	244	crandqgalerrfsvgdvadaavflasaesaalsgetievhgmelpac
SEQ ID NO:143	188	-----ltdia-----
SEQ ID NO:144	67	-----
SEQ ID NO:145	16	-----
SEQ ID NO:146	9	-----fpr-----
SEQ ID NO:147	192	-----amrr-----
SEQ ID NO:141	294	setsllartdlrtidasgrttlicagdqieevmaltgmrlrtcgseviigf
SEQ ID NO:143	193	-----
SEQ ID NO:144	67	-----dqikev-----
SEQ ID NO:145	16	-----
SEQ ID NO:146	12	-----qtqem-----
SEQ ID NO:147	196	-----ktvd-----
SEQ ID NO:141	344	rsaaalaqfeqavnesrrlagadftppialpldprpatidavfdwagen
SEQ ID NO:143	193	-----agsgfspdll-----ed
SEQ ID NO:144	73	-----
SEQ ID NO:145	16	-----qdrqpgieskmp-----
SEQ ID NO:146	17	-----pgttdrm-----
SEQ ID NO:147	200	-----

87/105

SEQ ID NO:141	394	tggihaavilpatshpapcvievdervlnfladeitgtivlasrlary
SEQ ID NO:143	205	tg-----ahtp-----
SEQ ID NO:144	73	-----lp-----
SEQ ID NO:145	29	-----lp-----
SEQ ID NO:146	24	-----qplp-----
SEQ ID NO:147	200	-----
SEQ ID NO:141	444	wqsqrtpgarargprviflsngadqngnvygriqsaaigqlirvrhea
SEQ ID NO:143	211	-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	31	-----lsededyrgs--gklk-----
SEQ ID NO:146	28	-----dhg-----
SEQ ID NO:147	200	-----
SEQ ID NO:141	494	eldygrasaagdhvlpvwanqivrfafrsleglefacawtaqlhsqrh
SEQ ID NO:143	211	-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	45	-----
SEQ ID NO:146	31	ensyqgsgrlkd-----
SEQ ID NO:147	200	-----
SEQ ID NO:141	544	ineitlnipanisattgarsasvgaesliglhlqkvalitggsagiggg
SEQ ID NO:143	211	-----lgkaa-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	45	-----gkvalitggsagigra-----
SEQ ID NO:146	43	-----kraitggsagigra-----
SEQ ID NO:147	200	-----nlpa-----
SEQ ID NO:141	594	igrllalsgarvmlaardrhk-leqmgamiqselaevgytdvedrvhiap
SEQ ID NO:143	216	-----qse-----
SEQ ID NO:144	73	-----
SEQ ID NO:145	61	aaiafakegadisilyldehsdaeetrkrieke-----nvrcilip
SEQ ID NO:146	58	vaiayaregadvlisylsehd-----damatkalve-----eagrkvlaa
SEQ ID NO:147	204	-----
SEQ ID NO:141	643	gcdvsseaqladlvertlsafgtvdylinnagiagveemvidmpvegwrh
SEQ ID NO:143	219	-----eiadmi-----
SEQ ID NO:144	73	-----vekvvqkygridvlvnagitr-dallvmkeedwda
SEQ ID NO:145	102	g-dvgdenhceqavqqtvdhfgkldilvnnaaeqhpqdsilnisteqlak
SEQ ID NO:146	99	g-diqssdhcrrivetavrelggidilvnnaahqatfkniedisdeewel
SEQ ID NO:147	204	-----eakaelkayvers-----
SEQ ID NO:141	693	tlfanlisnyslmrklaplmmkkqsgyilnvssyfggekdaaipypnrad
SEQ ID NO:143	225	-----
SEQ ID NO:144	109	vinvnlkgvfnvtqmvvpymikqngslvnvsevvg-----iynnpqgtn
SEQ ID NO:145	151	tftrnifsmfhtkklalphl--qegcainttsitayegdtal-----id
SEQ ID NO:146	148	tfrvnmhamfyltkaavphmkk-gsa-iintasi-----nadvnpilla
SEQ ID NO:147	217	-----
SEQ ID NO:141	743	yavskagqramaevfarfl-gpe-iginaiapgpvegdlrgtgerpglf
SEQ ID NO:143	225	-----
SEQ ID NO:144	154	yaaskagvigmtktwakelagrn-irvnavapgfie-----
SEQ ID NO:145	194	ysstkgaivsftrsmaksl-adkgirvnavapgpi-----
SEQ ID NO:146	191	yattkgaihnsaglaqml-aergirvnavapgpi-----
SEQ ID NO:147	217	yplgrigr-----

SUBSTITUTE SHEET (RULE 26)

88/105

SEQ ID NO:141	791	arrarlilenkrlnelhaaliaaartdersmhelvelllpndvaaleqnp
SEQ ID NO:143	225	-----
SEQ ID NO:144	189	-----
SEQ ID NO:145	228	-----wtp
SEQ ID NO:146	225	-----wtp lipstmpedtvadfgk
SEQ ID NO:147	225	-----pddlagm
SEQ ID NO:141	841	aaptalrelarrfrsegdpaassssallnrsiaakllarlhnggyvlpad
SEQ ID NO:143	225	-----
SEQ ID NO:144	189	-----
SEQ ID NO:145	231	lipatfpe-----
SEQ ID NO:146	244	qvp-----mkrggqpvelasa-----yvmld
SEQ ID NO:147	232	-----
SEQ ID NO:141	891	ifanlpnppdpfftraqidrearkvrdgimgmlylqrmptefdvamatvy
SEQ ID NO:143	225	-----vy
SEQ ID NO:144	189	-----
SEQ ID NO:145	239	-----ekvkq-----
SEQ ID NO:146	266	pmsy-----
SEQ ID NO:147	232	-----av
SEQ ID NO:141	941	yladrnvagetfhpssgglyertptggelfglpsperlaelvgstvyllg
SEQ ID NO:143	227	lasdk-----aksvtgsctyi--
SEQ ID NO:144	189	-----tpmteklpekareta--
SEQ ID NO:145	244	-----hgldtp-----
SEQ ID NO:146	271	-----vsgatiavtgg-----
SEQ ID NO:147	234	yia-----sdeawtsggi-----
SEQ ID NO:141	991	ehltehlnllaraylerygarqvmmivetetgaetmrrllhdhveagrlm
SEQ ID NO:143	242	-----
SEQ ID NO:144	204	-----lsriplgrfgkpe-----evaqvi
SEQ ID NO:145	250	-----
SEQ ID NO:146	282	-----
SEQ ID NO:147	248	-----
SEQ ID NO:141	1041	tivagdqieaaidqaitrygrpgpvvctpfrrlptvplvgrkdsdwstvl
SEQ ID NO:143	242	-----
SEQ ID NO:144	223	lflasdessyvtgqvi-----gidggglvi-----
SEQ ID NO:145	250	-----mgrpgqp-----
SEQ ID NO:146	282	-----kpfl-----
SEQ ID NO:147	248	-----favdgyt-----
SEQ ID NO:141	1091	seaefaelcehqlthhfrvarkialsdgalalvtpettatstteqfala
SEQ ID NO:143	242	-----mdnglalq-----
SEQ ID NO:144	247	-----
SEQ ID NO:145	258	-----eha-----gayvllasdes-----
SEQ ID NO:146	286	-----
SEQ ID NO:147	256	-----
SEQ ID NO:141	1141	nfikttlhaftatigvesertaqrilingvdltrraraeepdrpsherqqe
SEQ ID NO:143	250	-----
SEQ ID NO:144	247	-----
SEQ ID NO:145	272	-----syntgqtlhvn-----
SEQ ID NO:146	286	-----
SEQ ID NO:147	256	-----

SUBSTITUTE SHEET (RULE 26)

89/105

SEQ ID NO:141	1191	lerfieavllvtaplppeadtryagrihrgraitv
SEQ ID NO:143	250	-----
SEQ ID NO:144	247	-----
SEQ ID NO:145	283	-----ggrfist
SEQ ID NO:146	286	-----
SEQ ID NO:147	256	-----ag-----

SUBSTITUTE SHEET (RULE 26)

90/105

Figure 53

SEQ ID NO:140	1	atggcgacgggggagtcctatgagcggaaacaggacgactggcaggaaagat
SEQ ID NO:148	1	-----atga-----gacttctgcacaagcg
SEQ ID NO:149	1	-----atg-----ttcgcaataaagt
SEQ ID NO:150	1	-----atgaggcttgaagggaag--
SEQ ID NO:151	1	-----atggaaa--
SEQ ID NO:152	1	-----
SEQ ID NO:140	51	tgcgt-taattaccggtggcgccggcaatatcggcagtgaaattgacacgt
SEQ ID NO:148	21	cacgc-tggtgaccggcggtc-----
SEQ ID NO:149	18	ggtac-tagtaacaggtggttagctccggtatcggc-----
SEQ ID NO:150	20	tgtgtctgatcacagg---ggctgcaagcgggatagggaag-gccacca
SEQ ID NO:151	8	-----aatttccgca-----ccct
SEQ ID NO:152	1	-----
SEQ ID NO:140	100	cgctt--tctcgagagggagcgcgggtcattattagtgacgggaatcgg
SEQ ID NO:148	42	-----ggacgggtatcgg
SEQ ID NO:149	52	-----gcagctactgt-----
SEQ ID NO:150	65	cgcttcttttcgcacaggaag-----ga
SEQ ID NO:151	22	ccctt--tc-----
SEQ ID NO:152	1	-----
SEQ ID NO:140	148	gcgaagttgaccgcactggccgaacggatgcaggcagaggcaggagtgcc
SEQ ID NO:148	54	cc-----tggcaatcgccgagcggttccctgagcgagg-----
SEQ ID NO:149	63	-----ggaagcattc-----
SEQ ID NO:150	88	gctacggtgatcg--ctggc-----gat-----
SEQ ID NO:151	29	-----
SEQ ID NO:152	1	-----gtgaaccaatgg---acaga--caaacagaaggacaag-----
SEQ ID NO:140	198	ggcaaaagcgcacgatctcgaagtcattggatgggagtgatccgggtcgcg
SEQ ID NO:148	86	-----gcgc-----cgatgtcct-----
SEQ ID NO:149	73	-----gttaaggaagg-----
SEQ ID NO:150	109	-----atctcga-----
SEQ ID NO:151	29	-----
SEQ ID NO:152	35	-----aaccgcagc-----atcagg-----
SEQ ID NO:140	248	tacgtgccggtatcgaagcgatttgtggcccgctcacggccagatcgacatt
SEQ ID NO:148	99	-----gatcgctggccggtgacgcc-----
SEQ ID NO:149	84	-----cgcttctgtagccttcgtg-----
SEQ ID NO:150	116	-----aagaaaatctcgactct
SEQ ID NO:151	29	-----cccgcca-----
SEQ ID NO:152	50	-----acagacagccgggcatt
SEQ ID NO:140	298	ctggtcaacaatgcaggaagtgcgggtgccagcgtcgtctggccgagat
SEQ ID NO:148	118	-----gcc-----
SEQ ID NO:149	103	-----ggaagaaaccaagccaag-----
SEQ ID NO:150	133	cttgtgaaagaggcaggaag-----
SEQ ID NO:151	36	-----aaccaggaatgcc-----
SEQ ID NO:152	67	g-agtcaaaaatgaa-----tccgctgcc-----
SEQ ID NO:140	348	tccactcactgaagctgaattaggccctggcgccgaagagacgcttcattg
SEQ ID NO:148	121	-----aagct-----cgaagccgcgc-----g
SEQ ID NO:149	121	-----cttaag--gaagtag-----agagccgc-----tg
SEQ ID NO:150	153	-----
SEQ ID NO:151	51	-----
SEQ ID NO:152	90	-----

91/105

SEQ ID NO:140	398	ccagcatcgccaatttacttgggtatgggatggcatctgatgcgtattgcg
SEQ ID NO:148	138	ccagaagc-----tggcg
SEQ ID NO:149	144	ccagcagc-----
SEQ ID NO:150	153	-----actt-----
SEQ ID NO:151	51	-----cg
SEQ ID NO:152	90	-----gctgtcagaggacgaggattatc
SEQ ID NO:140	448	gcacctcatatgccggtaggaagtgcggtcatcaatgtctcgaccatctt
SEQ ID NO:148	151	gc-----tcttgcca-----
SEQ ID NO:149	152	-----atggagccaacatc-----
SEQ ID NO:150	157	-----cggg-ggaag-----
SEQ ID NO:151	53	gcac-----
SEQ ID NO:152	113	g-----aggaa-----
SEQ ID NO:140	498	ttcacgggctgagtactacgggaggattccgtatgtcaccacctaaagctg
SEQ ID NO:148	162	-----ggc-----
SEQ ID NO:149	166	-----ctggctatcaaag-----cagatgtctcc-----aaag-----
SEQ ID NO:150	166	-----
SEQ ID NO:151	57	-----tac-cgatcggatgc-----atgccg
SEQ ID NO:152	119	-----gcgg-----aaaactg
SEQ ID NO:140	548	ctcttaatgctctatctcaacttgctgcgctgagttaggtgcacgtggc
SEQ ID NO:148	165	-----cggcgc-----ggtggagacgtc
SEQ ID NO:149	194	-----acgagga
SEQ ID NO:150	166	-----
SEQ ID NO:151	76	c-----tgcccgat-----cacgggg-
SEQ ID NO:152	130	aaaggaa-----aagttg
SEQ ID NO:140	598	atccgcgttaatacagatctttcccgcccgattgaaagtgcacgtccg
SEQ ID NO:148	183	gtccgc-----cgatcttgcc-----
SEQ ID NO:149	201	agc-----gaaaatcatcgta-----
SEQ ID NO:150	166	-----gttgatccctacgtt-----ttgaacgtgaccg-----
SEQ ID NO:151	92	-----aaaac-----tcct
SEQ ID NO:152	143	-----cgatcattactgg-----
SEQ ID NO:140	648	tacagtgttcacgcgtatggatcagctcaagggggcgccgaaggcgaca
SEQ ID NO:148	199	-----
SEQ ID NO:149	217	-----
SEQ ID NO:150	194	-acag-----ggatcagataaag-----gaag-----
SEQ ID NO:151	101	accagggttcc-----ggacgcctgaag-----
SEQ ID NO:152	156	-----aggcgaca
SEQ ID NO:140	698	cagcgaccatttttgaacaccatgcgattgtgtcgtgccacgaccag
SEQ ID NO:148	199	-----accag
SEQ ID NO:149	217	-----caacaa-----
SEQ ID NO:150	215	-----ttgtggaaaa-----agtcttcaaa-----ag
SEQ ID NO:151	124	-----gacaag
SEQ ID NO:152	164	-----
SEQ ID NO:140	748	ggcgcgcttgaacgtcggttccctccgctcggtgatgtggcagacgcccgc
SEQ ID NO:148	204	-----cct-----
SEQ ID NO:149	223	-----ac
SEQ ID NO:150	238	tacg-----gtcgaatc-----gatgt-----
SEQ ID NO:151	130	agagc-----catcatcacggcgggga-----cagcggcatc
SEQ ID NO:152	164	-----

SUBSTITUTE SHEET (RULE 26)

92/105

SEQ ID NO:140	798	tgtctttctggccagtgccgaatccgcgctctctccggtgagacgattg
SEQ ID NO:148	207	-----cggtgtcgcaaccgtcg-tcgagcaggtgaaa-----
SEQ ID NO:149	225	tgtc-----gacaagttc-----gggaagcttg
SEQ ID NO:150	255	-----tctggtga-----
SEQ ID NO:151	163	gg-----cagggccgtggcga-----tcgcc-----
SEQ ID NO:152	164	-----
SEQ ID NO:140	848	aggttacgcacggaatggagttgccggcctgcagtgagaccagcctgctg
SEQ ID NO:148	238	-----gagaccggcc-----
SEQ ID NO:149	248	atgt-----
SEQ ID NO:150	263	-----
SEQ ID NO:151	184	-----tatgcgcgcgaggag-----c
SEQ ID NO:152	164	-----gcggaat-----agggagagc-----
SEQ ID NO:140	898	gcccgctactgatctgcgcacgattgatgccagtgcccgccacgacgctcat
SEQ ID NO:148	248	-----ggccgctcgcacattcct
SEQ ID NO:149	252	-----gcttggt-----aacaacgc-----
SEQ ID NO:150	263	-----acaacgc-----
SEQ ID NO:151	201	ggacgtccttatcagc-----tat
SEQ ID NO:152	180	-----
SEQ ID NO:140	948	ctgcgcggcgaccagattgaagaggtgatggcgctcacccggtatgttg
SEQ ID NO:148	265	.at-----caacaatg-----coggt-----
SEQ ID NO:149	267	-----
SEQ ID NO:150	270	-----
SEQ ID NO:151	220	ctgag-----cgagcatgacgacgcgagtgccaccaaggct-----
SEQ ID NO:152	180	-----
SEQ ID NO:140	998	gtacctgtgggagtgaagtgatcatcggttcoggtcggctcggcgctg
SEQ ID NO:148	280	-----gtcgccgacctc
SEQ ID NO:149	267	-----tgggatt-----ctacgggttcg-----
SEQ ID NO:150	270	-----gggaat-----
SEQ ID NO:151	256	-----ctggtggag-gaag-----
SEQ ID NO:152	180	-----
SEQ ID NO:140	1048	gcccgagttcgagcaggcagtcgaatgagagtcggcggtggccggcgcgaga
SEQ ID NO:148	292	gtgccggttcga-----gagcgtcagcg-----aggcgca-----
SEQ ID NO:149	284	-----cgagtgt-----tctggagccga
SEQ ID NO:150	276	-----
SEQ ID NO:151	269	-----caggtcgc-aaggcgt-----gcttgcgcggcgga
SEQ ID NO:152	180	-----agcag-----
SEQ ID NO:140	1098	ctttacgcctcccattgccttgccactcgatccacgcgatccggcaacaa
SEQ ID NO:148	321	-----gttcagcactcc
SEQ ID NO:149	302	cttta-----ataca-----aactt
SEQ ID NO:150	276	-----aacaa
SEQ ID NO:151	300	c-----atccagtcg-tccg-----acca
SEQ ID NO:152	185	-----ctattgcctt-----
SEQ ID NO:140	1148	ttgacgctg--tcttcgattgggcccggcgagaataccggcgggattcatg
SEQ ID NO:148	334	ttcgcgctc-----aatgtggcgg-----cggcg-----
SEQ ID NO:149	317	ttga-----
SEQ ID NO:150	281	gggatgc-----gcttcttg
SEQ ID NO:151	318	ttgccgcaggatcgtcgaaacggcgttcgggaactcggcgcat-----
SEQ ID NO:152	195	-----

93/105

SEQ ID NO:140 1196 cagcgtgattctgcctgctaccagtcacgaaccggcaccgtgcgtgatt
SEQ ID NO:148 358 -----ttcttcct-----cacc-----
SEQ ID NO:149 321 -----tgaaact-----
SEQ ID NO:150 296 -----
SEQ ID NO:151 363 -----
SEQ ID NO:152 195 -----tgcta-----

SEQ ID NO:140 1246 gaggttgatgatgagcgggtgctgaattttctggccgatgaaatcacccg
SEQ ID NO:148 370 -----caggggctgctgccgcatTT-----
SEQ ID NO:149 328 -----atgaac-----acgaatttac--g
SEQ ID NO:150 296 -----tgag-----gatgaaa-----c
SEQ ID NO:151 363 -----
SEQ ID NO:152 200 -----aagagggggctga-----

SEQ ID NO:140 1296 gacaattgtgattgccagtcgcctggcccggttactggcagtcgcaacggc
SEQ ID NO:148 390 -----
SEQ ID NO:149 345 tccagttgtcctcatcactagcctg-----
SEQ ID NO:150 307 -----
SEQ ID NO:151 364 gaca-----
SEQ ID NO:152 213 -----

SEQ ID NO:140 1346 ttacccccggcgacgtgcgcgtggggcgcgtgtcatttttctctcgaac
SEQ ID NO:148 390 -----cggcgc-----c
SEQ ID NO:149 370 -----
SEQ ID NO:150 307 -----
SEQ ID NO:151 368 -----ttctcgtcaac
SEQ ID NO:152 213 -----tatctccattctat--ac

SEQ ID NO:140 1396 ggtgccgatcaaaatgggaatgtttacggacgcattcaaagtgcgcgtat
SEQ ID NO:148 397 ggtgc-----at
SEQ ID NO:149 370 -----gctat
SEQ ID NO:150 307 -----gaagaagactgggatg-----
SEQ ID NO:151 379 aatgc-----
SEQ ID NO:152 229 ttagacgagca-----ttcggacgca-----

SEQ ID NO:140 1446 cggtcagctcattcgtgtgtggcgtcacgaggctgaacttgactatcagc
SEQ ID NO:148 404 cgatca-----
SEQ ID NO:149 375 ccctcatttgatt-----gctacaaaaggag-----
SEQ ID NO:150 323 cggT-----aataaac
SEQ ID NO:151 384 -----
SEQ ID NO:152 250 -----gagg-----aaac

SEQ ID NO:140 1496 gtgccagcgcgcgggtgatcatgtgctgccgcgggtatgggccaatcag
SEQ ID NO:148 410 -----
SEQ ID NO:149 402 -----
SEQ ID NO:150 334 gtg-----aatc--
SEQ ID NO:151 384 -----agcccatcag
SEQ ID NO:152 258 acgcaaacy-----gatc-----gaaaaggag

SEQ ID NO:140 1546 attgtgcgcttcgctaaccgcagccttgaagggttagaatttgctgtgc
SEQ ID NO:148 410 -----
SEQ ID NO:149 402 -----
SEQ ID NO:150 341 -----tgaagggt-----
SEQ ID NO:151 394 -----gcgaccttcaag-----
SEQ ID NO:152 280 aatgtccgctgc-----ctgcttatcc

SUBSTITUTE SHEET (RULE 26)

94/105

SEQ ID NO:140 1596 ctggacagctcaattgctccatagtcacgccatatcaatgagattaccc
SEQ ID NO:148 410 -----
SEQ ID NO:149 402 -----catagttaacg---tatccagtata-----
SEQ ID NO:150 349 -----gttttcaacg-----
SEQ ID NO:151 406 -----
SEQ ID NO:152 302 cggga-----

SEQ ID NO:140 1646 tcaacatccctgccaacattagcgccaccacggcgccagtcagtcg
SEQ ID NO:148 410 tcaacatctcttctattt-----cgcccgca-----
SEQ ID NO:149 424 -----ctgtctacaatag-----
SEQ ID NO:150 359 -----
SEQ ID NO:151 406 --aacatc---gaagacatcagcgac-----
SEQ ID NO:152 307 -----

SEQ ID NO:140 1696 gtcggatggcggaagcctgatcggttgcatctgggaaagtgcctt
SEQ ID NO:148 437 -----
SEQ ID NO:149 437 -----
SEQ ID NO:150 359 -----
SEQ ID NO:151 427 -----gagga-----
SEQ ID NO:152 307 ---gatg-----ttgggga-----

SEQ ID NO:140 1746 gattaccggtggcagcgccggtattggtggcagatcggcgccctcctgg
SEQ ID NO:148 437 -----
SEQ ID NO:149 437 -----
SEQ ID NO:150 359 -----
SEQ ID NO:151 432 -----gtggg-----
SEQ ID NO:152 318 -----

SEQ ID NO:140 1796 ctttgagtggcgcgcggtgatgctggcagccggtgatcggcataagctc
SEQ ID NO:148 437 -----
SEQ ID NO:149 437 -----taa-----
SEQ ID NO:150 359 -----
SEQ ID NO:151 437 -----agctgacattccg-----c
SEQ ID NO:152 318 -----

SEQ ID NO:140 1846 gaacagatgcaggcgatgatccaatctgagctggctgaggtgggtatac
SEQ ID NO:148 437 -----agatgatcc-----
SEQ ID NO:149 440 -----gaatac-----
SEQ ID NO:150 359 -----tgactcagatgg-----
SEQ ID NO:151 451 gtcaacatgcacgcatgttc-----tac
SEQ ID NO:152 318 -----cga-gaaccattgtgaacaagctg-----

SEQ ID NO:140 1896 cgatgtcgaagatcgcgctccacattgcaccgggctcgatgtgagtagcg
SEQ ID NO:148 446 -----cg
SEQ ID NO:149 446 c-----
SEQ ID NO:150 371 -----
SEQ ID NO:151 475 c--tgaccaag-----gcagcgg-----
SEQ ID NO:152 341 -----tgca-----

SEQ ID NO:140 1946 aagcgcagcttgcggtatctgttgaaacgtaccctgtcagcttttggcacc
SEQ ID NO:148 448 aagcg-----gccatc-----cagc
SEQ ID NO:149 447 -----
SEQ ID NO:150 371 -----
SEQ ID NO:151 491 -----tgccgcacatgaagaa-----gggcagc
SEQ ID NO:152 345 ----gcaaacagtggacc-----atcttggtaaa

95/105

SEQ ID NO:140 1996 gtcgattatctga-tcaacaacgccgggatcgccggtgtcgaagagatgg
SEQ ID NO:148 463 gtctactccctgt-ccaagggcgc-----
SEQ ID NO:149 447 -----
SEQ ID NO:150 371 -----
SEQ ID NO:151 514 g-----cga-tcatcaacacgc-----
SEQ ID NO:152 370 ctcgat-atcttagtgaacaacgcgc-----

SEQ ID NO:140 2045 ttatcgatatgccagttgagggatggcgccataccctcttcgccaatctg
SEQ ID NO:148 486 -----gttga-----
SEQ ID NO:149 447 -----aggattatgtcatacagt-----
SEQ ID NO:150 371 -----
SEQ ID NO:151 530 -----ctcca-----tcaatgccgacgttcccaatccg
SEQ ID NO:152 395 -----ctg

SEQ ID NO:140 2095 atcagcaactactcgttgatgcgcaaacctggcgccgttgatgaaaaaca
SEQ ID NO:148 491 -----actcgttga-----
SEQ ID NO:149 466 -----
SEQ ID NO:150 371 -----tggtgccctacatgatcaaaaca
SEQ ID NO:151 559 atc-----ctactcgcctatgcg-----accacca
SEQ ID NO:152 398 aacagcatc-----ccca

SEQ ID NO:140 2145 gggtagcgggttacatccttaacgtctcatcatactttggcgggtgaaaaag
SEQ ID NO:148 500 -----
SEQ ID NO:149 466 -----
SEQ ID NO:150 393 gaggaacggttcgatcgtgaacgtctcctctgtcgttgg-----aat
SEQ ID NO:151 584 agggcgcg-----atc-----cacaattt-----
SEQ ID NO:152 411 ggacag-----cattctcaatacttcaaca-----

SEQ ID NO:140 2195 atgcggccattccctaccccaaccgtgcgcgattacgcgctctcgaaggct
SEQ ID NO:148 500 -----ccagatcgct
SEQ ID NO:149 466 -----gtgtcaaaggct
SEQ ID NO:150 435 atacgggaat-----cctggtcagacgaattacggcggtcgaaggcg
SEQ ID NO:151 603 -----cagcgccg-----gtctcg-----
SEQ ID NO:152 436 -----

SEQ ID NO:140 2245 ggtcagcgggcaatggcgaagtctttgcgcgcttcttggcccg---ga
SEQ ID NO:148 510 ggccttcgag-----ctcggcccgcgcg
SEQ ID NO:149 478 g-----
SEQ ID NO:150 478 ggagtcataaggaatgacc-aagacgt-----
SEQ ID NO:151 617 -----cgcagatgctggccgaa-----cgcg---g-
SEQ ID NO:152 436 gaacagctggaa-----aaacctttcgc-----

SEQ ID NO:140 2292 gatacagatcaatgccattgcgcgggtccggtcgaagggtgatcgcttgc
SEQ ID NO:148 534 catccgcgtcaacgccatcgcgcccgccacggtcga-----
SEQ ID NO:149 479 -----
SEQ ID NO:150 503 -----ggcggaaggaaactcgct---
SEQ ID NO:151 639 gataagagtgaatgctgtggccccgggccccgac-----
SEQ ID NO:152 460 -acaaatattttttccat-----

SEQ ID NO:140 2342 gcggtaccggtgaacgtcccgccctctttgcccgctcgggcgcggtgatt
SEQ ID NO:148 570 -----
SEQ ID NO:149 479 -----
SEQ ID NO:150 520 -----
SEQ ID NO:151 673 -----tggaacgcgctg-----
SEQ ID NO:152 477 -----

SUBSTITUTE SHEET (RULE 26)

96/105

SEQ ID NO:140	2392	ttggagaacaagcggctgaatgagcttcacgctgctcttatcgcggtgc
SEQ ID NO:148	570	-----
SEQ ID NO:149	479	-----
SEQ ID NO:150	520	-----ggaagaacaatcaggggtgaac-----gctgt
SEQ ID NO:151	685	-----atccctccaccatgc-----
SEQ ID NO:152	477	-----gtttca-----
SEQ ID NO:140	2442	gcgaccgatgagcgatctatgcacgaactggttgaactgctcttacc
SEQ ID NO:148	570	-----
SEQ ID NO:149	479	-----ctatg---gatcacttcacaaat-----
SEQ ID NO:150	546	g-gcacc-----cgga
SEQ ID NO:151	701	-----ccgagga-----
SEQ ID NO:152	483	-----tatg-acgaa-----
SEQ ID NO:140	2492	atgatgtggccgcactagagcagaatccgcagcacctaccggttgcgt
SEQ ID NO:148	570	-----cacc-----
SEQ ID NO:149	500	-----tggcagcgttggagctg-----gctccttctggcgtgcga
SEQ ID NO:150	556	ttcat-----agaaaccccatgac-----
SEQ ID NO:151	708	-----taccg-----
SEQ ID NO:152	492	-----gaaagctttgcct-----
SEQ ID NO:140	2542	gaactggcagcagcttttcgcagcgaaggcgatccggcgcatcatcaag
SEQ ID NO:148	574	-----gccatgcggcg-----caag
SEQ ID NO:149	535	g-----
SEQ ID NO:150	576	-----cgaaaaacttccag-----aaaaag
SEQ ID NO:151	713	-----tcgcgatttcg-----caag
SEQ ID NO:152	505	cacctg-----
SEQ ID NO:140	2592	cagtgcgctgctgaaccgttcaattgcccgtataattgctggctcgtttgc
SEQ ID NO:148	589	-----accgt-----
SEQ ID NO:149	536	-----tgaac---tcagt-----
SEQ ID NO:150	596	c-----ccgtgaaacggcc-----gc
SEQ ID NO:151	725	-----
SEQ ID NO:152	515	aggggtg-----tgccatta-----
SEQ ID NO:140	2642	ataatggtggtatgtgttgctgcccacatctttgcacacctgccaaac
SEQ ID NO:148	594	-----cgac-----aacctgcc-----
SEQ ID NO:149	546	-----caaccctg-----
SEQ ID NO:150	610	-----ctttccaga-----
SEQ ID NO:151	727	aaacaggtgcctatg-----
SEQ ID NO:152	530	ttaat-----acgacat-----
SEQ ID NO:140	2692	ccgcccgatcccttcttcacccgagccagattgatcgcgaggctcgcaa
SEQ ID NO:148	606	-----
SEQ ID NO:149	554	-----gaccagttct-----
SEQ ID NO:150	619	-----atacc-----gctgggaa
SEQ ID NO:151	742	-----aa
SEQ ID NO:152	542	-----cgattaccgctt-----
SEQ ID NO:140	2742	ggttcgtgacggcatcatggggatgctctacctgcaacggatgccgactg
SEQ ID NO:148	606	-----ggccga-----
SEQ ID NO:149	564	-----tac-----
SEQ ID NO:150	632	ggtttgggaagccagaagagg-----
SEQ ID NO:151	744	g-----
SEQ ID NO:152	554	-----atgaaggggat-----acgg-----

97/105

SEQ ID NO:140 2792 agtttgatgtcgcaatggccaccgtctattaccttgccgaccgcaatgtc
SEQ ID NO:148 612 -----ggcca-----agggcgaactgaaggcc
SEQ ID NO:149 567 ----tgatatcgc-----
SEQ ID NO:150 653 -----tggcgca-----
SEQ ID NO:151 745 -----cgaccg-----
SEQ ID NO:152 569 -----cgttaattgattattccagcacaag-----

SEQ ID NO:140 2842 agtggtgagaca-ttccacccatcagggtgttgcggttacgaacgcaccc
SEQ ID NO:148 634 tatg-----tcgaacgcagc-----
SEQ ID NO:149 576 -----
SEQ ID NO:150 660 ---ggttatactcttccctcgcatcggacgagtcgagttacg-----
SEQ ID NO:151 751 -----
SEQ ID NO:152 595 ---ggtgcga-----ttgtttcctttacg-----

SEQ ID NO:140 2891 ctaccggtggcgaactcttcggcttgccctcaccggaacggctggcgagg
SEQ ID NO:148 649 -----tatccgctgggcccgcacgga-cggtccggacgac
SEQ ID NO:149 576 -----ag
SEQ ID NO:150 698 -----tcaccggacagg-----
SEQ ID NO:151 751 -----ggccagccc-----gtggaa
SEQ ID NO:152 616 cgttccatggcgaagtc---gcttgc-----

SEQ ID NO:140 2941 ctggtcggaagcacggtctatctgatagggaacatctgactgaacacct
SEQ ID NO:148 682 ctgcgcggcatggcggtttatct-----
SEQ ID NO:149 578 ctggt-----tctggct-----
SEQ ID NO:150 710 -----tgatag-----
SEQ ID NO:151 766 ctcg-----cctcgccctatgtcat-----
SEQ ID NO:152 639 -----agataaa-----

SEQ ID NO:140 2991 taacctgcttgcccgctgcgtacctcgaaacttacggggcacgtcaggtag
SEQ ID NO:148 705 -----
SEQ ID NO:149 590 -----
SEQ ID NO:150 716 -----
SEQ ID NO:151 786 -----
SEQ ID NO:152 646 -----ggca-----

SEQ ID NO:140 3041 tgatgattgttgagacagaaaccggggcagagacaatgcgtcgcttgctc
SEQ ID NO:148 705 -----
SEQ ID NO:149 590 -----tttctc-----
SEQ ID NO:150 716 -----
SEQ ID NO:151 786 -----
SEQ ID NO:152 650 -----tcagagtgaatgcg-----

SEQ ID NO:140 3091 caccgatcacgtcgaggctggtcggtgatgactattgtggccggtgatca
SEQ ID NO:148 705 -----
SEQ ID NO:149 596 c-----tgatct-----
SEQ ID NO:150 716 -----
SEQ ID NO:151 786 -----gctgg-----
SEQ ID NO:152 664 -----gtggcgcccggt-----

SEQ ID NO:140 3141 gatcgaagccgctatcgaccaggctatcactcgctacggtcgccagggc
SEQ ID NO:148 705 -----agccagcgacgaggc-----
SEQ ID NO:149 603 gcttgaag-----
SEQ ID NO:150 716 -----
SEQ ID NO:151 791 ---cggatccgatgtcga-----gctac-----
SEQ ID NO:152 676 -----ccgatttgacaccgct-----

SUBSTITUTE SHEET (RULE 26)

98/105

SEQ ID NO:140 3191 cggtcgtctgtacccccctccggccactgccgacggtaccactggtcggg
SEQ ID NO:148 720 -----
SEQ ID NO:149 611 -----
SEQ ID NO:150 716 -----
SEQ ID NO:151 811 -----
SEQ ID NO:152 693 -----tattccgg-----cgacattccctgagg-----

SEQ ID NO:140 3241 cgtaaagacagtgactggagcacagtgttgagtgaggctgaatttgccga
SEQ ID NO:148 720 -----ggcctgga-----cga
SEQ ID NO:149 611 -----atacaggg-----
SEQ ID NO:150 716 -----gaat-----
SEQ ID NO:151 811 -----gtgtcaggcgca-----
SEQ ID NO:152 716 -----aaaaagtga-aacagcac-----ggcttgatacccca

SEQ ID NO:140 3291 gttgtgcgaacaccagctcaccacacatttccgggtagcgcaagattg
SEQ ID NO:148 731 gcggtgggatc-----tttg
SEQ ID NO:149 619 -----gctcatcacaccgt-----
SEQ ID NO:150 720 -----
SEQ ID NO:151 823 -----acgattg
SEQ ID NO:152 748 ---atgggaagaccgggacagcc-----ggttgagc-----

SEQ ID NO:140 3341 ccctgagtgatggtgc-cagtctcgcgctggtcactcccgaaactacggc
SEQ ID NO:148 746 ccgtg---gatggt-----
SEQ ID NO:149 632 ---tggggaagctgcgcagctc-----
SEQ ID NO:150 720 ---agatgg-----
SEQ ID NO:151 830 ccgtga-----
SEQ ID NO:152 776 -----atgcaggcgc-ctatgtctgctggcgtctgacgaa-----

SEQ ID NO:140 3390 tacctcaactaccgagcaatttgcctctggttaacttcatcaaaacgaccc
SEQ ID NO:148 757 -----
SEQ ID NO:149 652 -----gaggagattgct-----
SEQ ID NO:150 726 -----
SEQ ID NO:151 836 -----
SEQ ID NO:152 811 -----tcttccta-----

SEQ ID NO:140 3440 ttcacgcttttacggctacgattggtgtcgagagcgaaagaactgctcag
SEQ ID NO:148 757 -----ggcta-----
SEQ ID NO:149 664 -----gatatgatt-----
SEQ ID NO:150 726 -----
SEQ ID NO:151 836 -----
SEQ ID NO:152 819 -----tatga-----cag

SEQ ID NO:140 3490 cgcattctgatcaatcaagtcatctgacccggcggtgcggtgccgaaga
SEQ ID NO:148 762 -----
SEQ ID NO:149 673 -----gtgtatctg-----gctagtataaagc
SEQ ID NO:150 726 -----gg
SEQ ID NO:151 836 -----
SEQ ID NO:152 827 ggca---gaccattcatgt-----gaatg

SEQ ID NO:140 3540 gccgcgtgatccgcacgagcgtcaacaagaactggaacgttttatcgagg
SEQ ID NO:148 762 -----
SEQ ID NO:149 696 taagagtgtt-----acggggtcctgttat-----
SEQ ID NO:150 728 gctcgtgat-----
SEQ ID NO:151 836 -----
SEQ ID NO:152 848 gcggc-----cgttttat-----

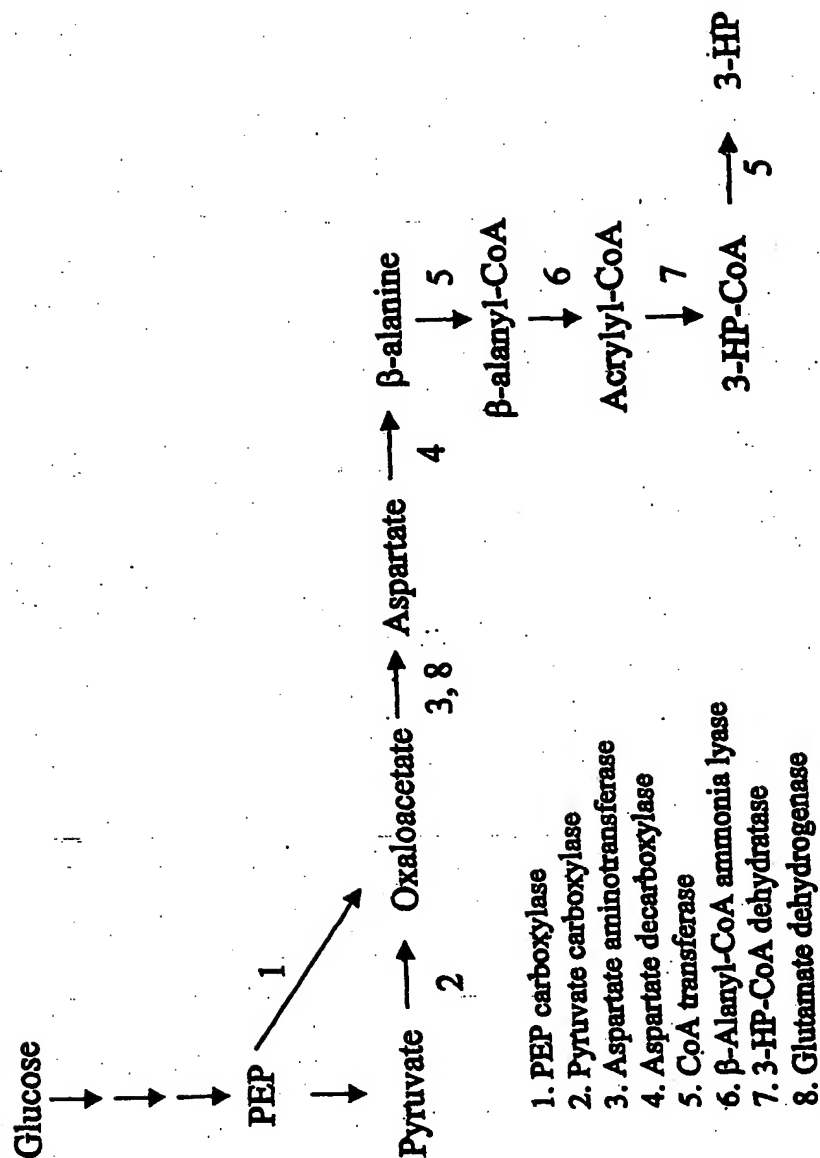
99/105

SEQ ID NO:140	3590	cagtccttgctggtcactgcaccactcccgctgaagccgatacccgttac
SEQ ID NO:148	762	-----
SEQ ID NO:149	721	----atcatggacaatg---gactcgcgc-----
SEQ ID NO:150	738	-----ctga-----
SEQ ID NO:151	836	-----ccggcggcaagcc-----
SEQ ID NO:152	861	-----
SEQ ID NO:140	3640	gccgggaggattcatcgcgacgggagattaccgtgtaa .
SEQ ID NO:148	762	-----cacggccggatga-----
SEQ ID NO:149	743	-----tgca-----gtaa
SEQ ID NO:150	742	-----
SEQ ID NO:151	849	-----tttcctttga-
SEQ ID NO:152	861	-----ttcaac-----gtaa

SUBSTITUTE SHEET (RULE 26)

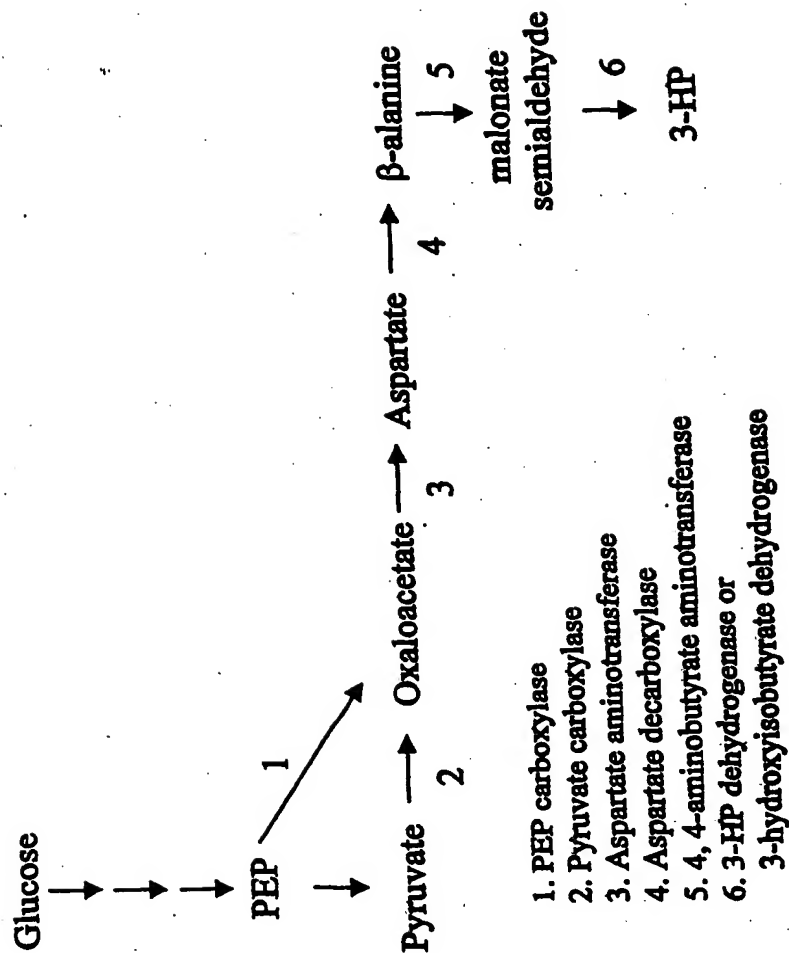
100/105

Figure 54



101/105

Figure 55



SUBSTITUTE SHEET (RULE 26)

102/105

Figure 56

1 MVGKKVVHHL MMSAKDAHYT GNLVNGARIV NQWGDVGTEL
41 MVIYVDGDISL FLGYKDIEFT APVYVGDFME YHGWIKEVGN
81 QSYTCKFEAW KVATMVDITN PQDTRATACE PPVLCGRATG
121 SLFIAKKDQR GPQESSFKER KHPGE (SEQ ID NO:160)

103/105

Figure 57

1 MVGKKVVHHL MMSAKDAHYT GNLVNGARIV NOWGDVGTEL
41 MUYVDGDISL FLGYKDIEFT APVYVGDFME YHGWIEKVGN
81 QSYTCKFEAW KVAKMVDITN PQDTRATACE PPVLCGTATG
121 SLFIAKDNQR GPQESSFKDA KHPQ (SEQ ID NO:161)

SUBSTITUTE SHEET (RULE 26)

104/105

Figure 58

```
1  ATGGTAGGTA AAAAGGTTGT ACATCATTTA ATGATGAGCG
41  CAAAAGATGC TCACTATACT GGAAACTTAG TAAACGGGCGC
81  TAGAATTGTG AATCAGTGGG GCGACGTTGG TACAGAATTA
121 ATGGTTTATG TTGATGGTGA CATAAGCTTA TTCTTGGGCT
161 ACAAAGATAT CGAATTCACA GCTCCTGTAT ATGTTGGTGA
201 CTTTATGGAA TACCACGGCT GGATTGAAA AGTTGGTAAC
241 CAGTCCTATA CATGTAAATT TGAAGCATGG AAAGTTGCAA
281 CAATGGTTGA TATCACAAT CCTCAGGATA CACGCGCAAC
321 AGCTTGTGAG CCTCCGGTAT TGTGCGGAAG AGCAACGGGT
361 AGTTTGTTC TCGCAAAAA AGATCAGAGA GGCCCTCAGG
401 AATCCTCTTT TAAAGAGAGA AAGCACCCCG GTGAATGA
(SEQ ID NO:162)
```

105/105

Figure 59

```
1  ATGGTAGGTA AAAAGGTTGT ACATCATTTA ATGATGAGCG
41 CAAAAGATGC TCACTATACT GGAAACTTAG TAAACGGCGC
81 TAGAATTGTG AATCAGTGGG GCGACGTAGG TACAGAATTA
121 ATGGTTTATG TTGATGGTGA CATCAGCTTA TTCTTGGGCT
161 ACAAAGATAT CGAATTCACA GCTCCTGTAT ATGTTGGTGA
201 TTTTATGGAA TACCACGGCT GGATTGAAAA AGTTGGCAAC
241 CAGTCCTATA CATGTAAATT TGAAGCATGG AAAGTAGCAA
281 AGATGGTTGA TATCACAAT CCACAGGATA CACGTGCAAC
321 AGCTTGTGAA CCTCCGGTAC TTTGTGGTAC TGCAACAGGC
361 AGCCTTTTCA TCGCAAAGGA TAATCAGAGA GGTCTCAGG
401 AATCTTCCTT CAAGGATGCA AAGCACCTC AATAA
(SEQ ID NO:163)
```

SUBSTITUTE SHEET (RULE 26)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
30 May 2002 (30.05.2002)

PCT

(10) International Publication Number
WO 02/042418 A3

(51) International Patent Classification⁷: C12N 9/10,
9/14, 1/20, 15/00, C07H 21/04

(21) International Application Number: PCT/US01/43607

(22) International Filing Date:
20 November 2001 (20.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/252,123 20 November 2000 (20.11.2000) US
60/285,478 20 April 2001 (20.04.2001) US
60/306,727 20 July 2001 (20.07.2001) US
60/317,845 7 September 2001 (07.09.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US	60/252,123 (CON)
Filed on	20 November 2000 (20.11.2000)
US	60/285,478 (CON)
Filed on	20 April 2001 (20.04.2001)
US	60/306,727 (CON)
Filed on	20 July 2001 (20.07.2001)
US	60/317,845 (CON)
Filed on	7 September 2001 (07.09.2001)

(71) Applicant (for all designated States except US):
CARGILL, INCORPORATED [US/US]; 15407
McGinty Road West, Wayzata, MN 55391-2399 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **GOKARN, Ravi, R.** [IN/US]; 3205 Harbor, Lane #4311, Plymouth, MN 55447 (US). **SELIFONOVA, Olga, V.** [RU/US]; 1405 Olive Lane N. #318, Plymouth, MN 55447 (US). **JESSEN, Holly** [US/US]; 6618 Brenden Court, Chanhassen, MN 55317

(US). **GORT, Steven, J.** [US/US]; 3207 Quarles Road, Brooklyn Park, MN 55429 (US). **SELMER, Thorsten** [DE/DE]; Cappeler Strasse 12, 35039 Marburg (DE). **BUCKEL, Wolfgang** [DE/DE]; Am Koeppel 8, 35043 Marburg (DE).

(74) Agent: **DEGRANDIS, Paula**; Cargill, Incorporated, P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:
26 June 2003

(15) Information about Correction:
Previous Correction:

see PCT Gazette No. 47/2002 of 21 November 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/042418 A3

(54) Title: 3-HYDROXYPROPIONIC ACID AND OTHER ORGANIC COMPOUNDS

(57) Abstract: The invention provides methods and materials related to producing 3-HP as well as other organic compounds. Specifically, the invention provides isolated nucleic acids, polypeptides, host cells, and methods and materials for producing 3-HP and other organic compounds.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/43607

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 9/10, 9/14, 1/20, 15/00; C07H 21/04

US CL : 435/193, 195, 252.3, 320.1; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/193, 195, 252.3, 320.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN AND WEST, Sequence Search for SEQ ID No : 1 in US and commercial data bases**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,323,010 B1 (SKALY et al.) 27 November 2001 (27.11.2001), see the entire document.	1-42, 44-47

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier application or patent published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

20 March 2003 (20.03.2003)

Date of mailing of the international search report

10 APR 2003

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Telicia D. Roberts for
Tekchand Saluja

Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/43607

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-42 & 44-47 (All partly)

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US01/43607

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 1, host cell and the method of making the polypeptide.

Group II, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 9, host cell and the method of making the polypeptide.

Group III, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 17, host cell and the method of making the polypeptide.

Group IV, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 25, host cell and the method of making the polypeptide.

Group V, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 33, host cell and the method of making the polypeptide.

Group VI, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 34, host cell and the method of making the polypeptide.

Group VII, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 36, host cell and the method of making the polypeptide.

Group VIII, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 38, host cell and the method of making the polypeptide.

Group IX, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 40, host cell and the method of making the polypeptide.

Group X, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 42, host cell and the method of making the polypeptide.

Group XI, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 129, host cell and the method of making the polypeptide.

Group XII, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 140, host cell and the method of making the polypeptide.

Group XIII, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 142, host cell and the method of making the polypeptide.

Group XIV, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 162, host cell and the method of making the polypeptide.

Group XV, claim(s) 1-42 & 44-47 (Partially), drawn to nucleic acid sequence of SEQ ID NO : 163, host cell and the method of making the polypeptide.

Group XVI, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 2.

Group XVII, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 10.

Group XVIII, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 18.

Group XIX, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 26.

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US01/43607

- Group XX, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 35.
- Group XXI, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 37.
- Group XXII, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 39.
- Group XXIII, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 41.
- Group XXIV, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 141.
- Group XXV, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 160.
- Group XXVI, claim(s) 43 (Partially), drawn to binding agent to polypeptide of SEQ ID NO : 161.
- Group XXVII, claim(s) 48-59 & 78-90, drawn to a method of making 3-HP (3-hydroxypropionic acid).
- Group XXVIII, claim(s) 60-64, drawn to a method of making polymerized 3-HP (3-hydroxypropionic acid).
- Group XXIX, claim(s) 65-69, drawn to a method of making ester of 3-HP.
- Group XXX, claim(s) 70-73, drawn to a method of making polymerized acrylate.
- Group XXXI, claim(s) 74-77, drawn to a method of making ester of acrylate.

The inventions listed as Groups I-XXXI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I has a special technical feature of a nucleic acid of SEQ ID NO : 1, which Groups II-XXXI do not share. Group II has a special technical feature of a nucleic acid of SEQ ID NO : 9, which Groups I and III-XXXI do not share. Group III has a special technical feature of a nucleic acid of SEQ ID NO : 17, which Groups I-II and IV-XXXI do not share. Group IV has a special technical feature of a nucleic acid of SEQ ID NO : 25, which Groups I-III and V-XXXI do not share. Group V has a special technical feature of a nucleic acid of SEQ ID NO : 33, which Groups I-IV and VI-XXXI do not share. Group VI has a special technical feature of a nucleic acid of SEQ ID NO : 34, which Groups I-V and VII-XXXI do not share. Group VII has a special technical feature of a nucleic acid of SEQ ID NO : 36, which Groups I-VI and VIII-XXXI do not share. Group VIII has a special technical feature of a nucleic acid of SEQ ID NO : 38, which Groups I-VII and IX-XXXI do not share. Group IX has a special technical feature of a nucleic acid of SEQ ID NO : 40, which Groups I-VIII and X-XXXI do not share. Group X has a special technical feature of a nucleic acid of SEQ ID NO : 42, which Groups I-IX and XI-XXXI do not share. Group XI has a special technical feature of a nucleic acid of SEQ ID NO : 129, which Groups I-X and XII-XXXI do not share. Group XII has a special technical feature of a nucleic acid of SEQ ID NO : 140, which Groups I-XI and XIII-XXXI do not share. Group XIII has a special technical feature of a nucleic acid of SEQ ID NO : 142, which Groups I-XII and XIV-XXXI do not share. Group XIV has a special technical feature of a nucleic acid of SEQ ID NO : 162, which Groups I-XIII and XV-XXXI do not share. Group XV has a special technical feature of a nucleic acid of SEQ ID NO : 163, which Groups I-XIV and XVI-XXXI do not share. Group XVI has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 2, which Groups I-XV and XVII-XXXI do not share. Group XVII has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 10, which Groups I-XVI and XVIII-XXXI do not share. Group XVIII has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 18, which Groups I-XVII and XIX-XXXI do not share. Group XIX has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 26, which Groups I-XVIII and XX-XXXI do not share. Group XX has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 35, which Groups I-XIX and XXI-XXXI do not share. Group XXI has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 37, which Groups I-XX and XXII-XXXI do not share. Group XXII has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 39, which Groups I-XXI and XXIII-XXXI do not share. Group XXIII has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 41, which Groups I-XXII and XXIV-XXXI do not share. Group XXIV has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 141, which Groups I-XXIII and XXV-XXXI do not share. Group XXV has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 160, which Groups I-XXIV and XXVI-XXXI do not share. Group XXVI has a special technical feature of a binding agent to polypeptide of SEQ ID NO : 161, which Groups I-XXV and XXVII-XXXI do not share. Groups XXVII-XXXI are drawn to making of different products employing different method steps and end products and distinct among themselves as well with respect to the Groups I-XXVI which employ sequences that Groups XXVII-XXXI do not share.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.